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CARACTERIZAÇÃO QUÍMICA E AVALIAÇÃO DA TOXICIDADE E DO POTENCIAL ANTIMICROBIANO DO ÓLEO ESSENCIAL DE *Eugenia gracillima* Kiaersk. (Myrtaceae)

Recife
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Tese de Doutorado apresentada para o cumprimento parcial das exigências para obtenção do título de Doutora em Ciências Biológicas pela Universidade Federal de Pernambuco.

Orientadora: Profª. Drª. Márcia Vanusa da Silva.

Coorientador: Prof. Dr. Clovis Macêdo Bezerra Filho.

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Minha energia é o desafio,
minha motivação é o impossível,
e é por isso que eu preciso ser,
à força e a esmo, inabalável (BRANCO, 2010).

RESUMO

Considerando a necessidade do desenvolvimento de novos compostos antimicrobianos, este trabalho teve como objetivo identificar a composição química, a citotoxicidade e o potencial antimicrobiano do óleo essencial das folhas de *Eugenia gracillima* Kiaersk. (Myrtaceae). O óleo essencial de *E. gracillima* (EgEO) foi obtido através do método de hidrodestilação em aparelho Clevenger e analisado quimicamente através da cromatografia gasosa acoplada à espectrometria de massas (CG/EM). Todos os compostos identificados no EgEO pertencem a classe dos sesquiterpenos (97,02%). A citotoxicidade do EgEO avaliada em eritrócitos e em linhagens de células tumorais, demonstrou baixo percentual de hemólise nas concentrações testadas (% de hemólise: 0,29 – 10,47%) e elevado potencial inibitório para as linhagens de células tumorais (CI_{50}: 10,8 – 35,0 µg/mL). A atividade antimicrobiana do EgEO foi avaliada frente a linhagens padrão e multirresistentes dos patógenos oportunistas de interesse clínico *Staphylococcus aureus* e *Candida glabrata*. EgEO apresentou atividade inibitória contra todas as linhagens de *S. aureus* (CMI: 0,01 – 1,84 mg/mL) e *C. glabrata* (CMI: 0,92 – 3,68 mg/mL) testadas, atividade bactericida contra sete linhagens de *S. aureus* (CMB: 0,05 – 1,84 mg/mL) e atividade fungicida contra três linhagens de *C. glabrata* (CMF: 0,92 – 3,69 mg/mL). EgEO reduziu a biossíntese do pigmento carotenoide estafiloxantina (STX) nas linhagens de *S. aureus* testadas, sugerindo que o óleo possui também atividade antivirulência. Nos ensaios *in vivo*, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) foi utilizado como organismo modelo e a dose letal (DL) foi determinada para diferentes estágios de desenvolvimento do inseto. Além disso, o desenvolvimento de *T. molitor* foi avaliado após aplicação de doses sub-letais de EgEO em indivíduos no último estágio larval do ciclo de vida. EgEO apresentou baixa toxicidade (DL_{50}: 4,55 – 5,98 e DL_{90}: 13,60 – 17,10 mg/mL) e não induziu alterações morfológicas em *T. molitor*. O mesmo organismo foi utilizado como modelo de infecção *in vivo* para avaliar o potencial antimicrobiano do EgEO frente a *S. aureus* e *C. glabrata*. EgEO demontrou potencial para tratar a infecção causada por cepas padrão de *S. aureus* (Concentração de EgEO: 4,612 mg/mL) e *C. glabrata* (Concentração de EgEO: 2,302 mg/mL), sugerindo que os mecanismos de resistência desenvolvidos ao longo do tempo pelas linhagens clínicas sejam favoráveis nas condições *in vivo*. Este estudo demonstrou pela primeira vez que o óleo essencial das folhas de *E. gracillima* apresenta uma composição rica em sesquiterpenos, baixa toxicidade, além de potencial antitumoral e antimicrobiano frente a linhagens padrão e multirresistentes de patógenos oportunistas de interesse clínico.

Palavras-chave: Myrtaceae. Sesquiterpenos. Estafiloxantina. Antivirulência.

ABSTRACT

Considering the need for the development of new antimicrobial compounds, this work aimed to identify the chemical composition, cytotoxicity and antimicrobial potential of *Eugenia gracillima* Kiaersk. essential oil. (Myrtaceae). The *E. gracillima* essential oil (EgEO) was obtained from fresh leaves by the hydrodistillation method in Clevenger apparatus and analyzed chemically by gas chromatography coupled to mass spectrometry (GC/MS). All the compounds identified in EgEO belong to the sesquiterpene class (97.02%). The cytotoxicity of EgEO evaluated on erythrocytes and tumor cell lines demonstrated a low percentage of hemolysis at the concentrations tested (% hemolysis: 0.29 - 10.47%) and high inhibitory potential for the tumor cell lines (IC_{50}: 10.8 - 35.0 μg / ml). The antimicrobial activity of EgEO was evaluated against standard and multiresistant strains of opportunistic pathogens of clinical interest *Staphylococcus aureus* and *Candida glabrata*. EgEO showed inhibitory activity against all strains of *S. aureus* (MIC: 0.01 - 1.84 mg/mL) and *C. glabrata* (MIC: 0.92 - 3.68 mg/mL), bactericidal activity against seven *S. aureus* strains (MBC: 0.05 - 1.84 mg/mL) and fungicidal activity against three *C. glabrata* strains (MFC: 0.92 - 3.69 mg/mL). EgEO reduced the biosynthesis of carotenoid pigment staphyloxanthin (STX) in the strains of *S. aureus* tested, suggesting that the oil also has anti-virulence activity. In the *in vivo* tests, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) was used as a model organism and the lethal dose (LD) was determined for different stages of development of the insect. In addition, the development of *T. molitor* was evaluated after application of sublethal doses of EgEO in individuals in the last larval stage of the life cycle. EgEO presented low toxicity (LD_{50}: 4.55 - 5.98 and LD_{90}: 13.60 - 17.10 mg/mL) and did not induce morphological changes in *T. molitor*. The same organism was used as an *in vivo* infection model to evaluate the antimicrobial potential of EgEO against *S. aureus* and *C. glabrata*. EgEO demonstrated potential to treat infection caused by the standard strains of *S. aureus* (EgEO concentration: 4.612 mg/mL) and *C. glabrata* (EgEO concentration: 2.302 mg/mL), suggesting that resistance mechanisms developed over time by the clinical strains are favorable in the *in vivo* conditions. This study demonstrated for the first time that the essential oil of the leaves of *E. gracillima* presents a composition rich in sesquiterpenes, low toxicity, besides antitumor and antimicrobial potential against standard and multiresistant strains of opportunistic pathogens of clinical interest.

Keywords: Myrtaceae. Sesquiterpenes. Staphyloxanthin. Anti-virulence.

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LISTA DE SIGLAS

ASD	Ágar Sabouraud Dextrose
CA-MRSA	*Staphylococcus aureus* resistente à meticilina adquirido na comunidade
CLSI	Clinical & Laboratory Standards Institute
CMB	Concentração Mínima Bactericida
CMF	Concentração Mínima Fungicida
CMI	Concentração Mínima Inibitória
CTE	Cadeia Transportadora de Elétrons
DMAPP	Difosfato de Dimetilalila
DNA	Ácido Desoxirribonucleico
EgEO	Óleo Essencial de *Eugenia gracillima* Kiaersk.
GGPP	Difosfato de Geranil Geranila
GPP	Difosfato de Geranila
HA-MRSA	*Staphylococcus aureus* resistente à meticilina adquirido em serviços de assistência à saúde.
IgG	Imunoglobulina G
IPP	Difosfato de Isopentenila
LB	Luria-Bertani
LPSN	List of Prokaryotic Names with Standing in Nomenclature
MEP	Metileritritol-fosfato
MEV	Mevalonato
MH	Muller-Hinton
MHC	Major Histocompatibility Complex
MRSA	*Staphylococcus aureus* resistente à meticilina
OD	Optical Density
OEs	Óleos Essenciais
OMS	Organização Mundial de Saúde
PAL	Fenilalanina amônia-liase
RNA	Ácido Ribonucleico
ROS	Espécies Reativas de Oxigênio
STX	Estafiloxantina
TSCT	Toxina da Síndrome do Choque Tóxico

SUMÁRIO

1 INTRODUÇÃO

O surgimento acelerado de novos mecanismos de resistência microbiana tornou-se um problema de saúde pública mundial. A resistência aos antimicrobianos tem sido atribuída, principalmente, ao uso exacerbado e indevido desses fármacos (PADIYARA; INOUE; SPRENGER, 2018). O aumento da resistência microbiana tem levado a necessidade de novos fármacos e novas classes de antimicrobianos, tanto para infecções adquiridas em hospitais quanto na comunidade.

Staphylococcus aureus e *Candida glabrata* são considerados patógenos oportunistas relacionados a múltipla resistência aos antimicrobianos. *S. aureus* é um dos principais agentes causadores de bacteremia, endocardite infecciosa, infecções osteoarticulares, cutâneas, pleuropulmonares e infecções relacionadas a dispositivos médicos (ARYEE; EDGEWORTH, 2016). *C. glabrata* está entre os três principais agentes causadores de candidíase (CAVALHEIRO *et al*., 2019). O crescente perfil de resistência apresentado por estes patógenos torna necessário o desenvolvimento de novos fármacos para o tratamento das suas infecções.

Os óleos essenciais obtidos de plantas medicinais e aromáticas são compostos voláteis complexos. Algumas pesquisas sugerem que esses óleos essenciais apresentam potencial biológico, como por exemplo, antioxidante, anti-inflamatório, antitumoral e antimicrobiano (TEIXEIRA *et al*., 2013; ALI *et al*., 2015). As propriedades antimicrobianas dos óleos essenciais contra diversos agentes patogênicos bacterianos, fúngicos e virais, atribuí-se a presença de diferentes classes de compostos em sua composição, tais como, terpenos, fenóis e aldeídos (AKHTAR; DEGAGA; AZAM, 2014).

A família Myrtaceae abrange cerca de 132 gêneros com aproximadamente 5.950 espécies, tem como característica marcante a presença de glândulas oleíferas translúcidas nas folhas, flores e frutos, o que justifica a produção relativamente alta de óleos essenciais pelos seus representantes (WATSON; DALLWITZ, 2017; BIDA *et al*., 2018). Um dos maiores gêneros da família Myrtaceae é o *Eugenia*, cujas espécies produzem diversos metabólitos secundários com propriedades farmacológicas importantes (CANTANHEDE-FILHO *et al*., 2017).

Eugenia gracillima Kiaersk. (Myrtaceae) é uma espécie nativa e endêmica do Brasil. A planta é um arbusto variando em altura de 1,5 a 5,0 metros, conhecida popularmente como murta. No Nordeste, seus frutos são socioeconomicamente importantes, uma vez que são

consumidos frescos ou processados em forma de polpa, suco, géleia, licor ou sobremesas (ARAUJO *et al*., 2016).

Considerando os aspectos mencionados, este estudo investigou o perfil fitoquímico, a citotoxicidade e o potencial antimicrobiano do óleo essencial das folhas de *E. gracillima* Kiaersk., frente a cepas padrão e multirresistentes dos patógenos oportunistas *S. aureus* e *C. glabrata*.

1.1 OBJETIVOS

1.1.1 Geral

Extrair, avaliar e caracterizar o perfil fitoquímico, a toxicidade e o potencial antimicrobiano do óleo essencial das folhas de *E. gracillima* contra os patógenos oportunistas *S. aureus* e *C. glabrata*.

1.1.2 Específicos

- Extrair o óleo essencial a partir das folhas frescas de *E. gracillima* e caracterizar através de cromatografia gasosa acoplada à espectrometria de massas (CG/EM).
- Avaliar a atividade antimicrobiana *in vitro* do óleo essencial de *E. gracillima* (EgEO), determinando a concentração mínima inibitória (CMI), a concentração mínima bactericida (CMB) e a concentração mínima fungicida (CMF) frente a cepas padrão e multirresistentes de *S. aureus* e *C. glabrata.*
- Avaliar a cinética de crescimento de *S. aureus* e *C. glabrata* após exposição ao EgEO.
- Investigar o potencial antivirulência do EgEO através de alterações na biossíntese do pigmento estafiloxantina (STX) por *S. aureus*.
- Avaliar a toxicidade *in vivo* do EgEO utilizando *T. molitor* como organismo modelo.
- Avaliar a atividade hemolítica *in vitro* do EgEO.
- Avaliar a atividade citotóxica *in vitro* do EgEO frente a linhagens celulares tumorais.
- Avaliar o potencial antimicrobiano *in vivo* do EgEO utilizando *T. molitor* como organismo hospedeiro para *S. aureus* e *C. glabrata*.

2 REVISÃO BIBLIOGRÁFICA

2.1 RESISTÊNCIA AOS ANTIMICROBIANOS

A resistência aos antimicrobianos tornou-se um importante problema de saúde pública. Em 2014, o relatório global sobre resistência antimicrobiana da Organização Mundial de Saúde (OMS) considerou que o crescimento da resistência aos antimicrobianos é uma ameaça à saúde mundial (WHO, 2014). Do ponto de vista epidemiológico, microrganismos resistentes são aqueles que apresentam resistência a uma ou mais classes de antimicrobianos. Sob a perspectiva laboratorial, entende-se como o crescimento de um microrganismo *in vitro* na presença de drogas que comumente interfeririam em suas funções de crescimento, as quais seriam habitualmente sensíveis (CLSI, 2015).

Esse mecanismo é um processo natural observado desde a descoberta dos primeiros antibióticos. Estudos demonstraram que, de fato, os genes que conferem resistência a drogas em algumas cepas de microrganismos pré-datam os antibióticos em milhões de anos. Embora a resistência seja um processo evolutivo normal para os microrganismos, ela foi acelerada pela pressão seletiva exercida pelo uso generalizado de drogas antibióticas. A resistência aos antimicrobianos como problema de saúde pública emergiu como consequência do uso indiscriminado pela população e das prescrições inapropriadas (VENTOLA, 2015).

Existem duas estratégias genéticas gerais de resistência. Uma compreende mecanismos que transferem resistência verticalmente de um microrganismo para sua progênie, tais como mutações em genes cromossômicos. A segunda consiste na tranferência de genes horizontalmente para outros microrganismos, até mesmo entre os de diferentes gêneros. Elementos genéticos móveis, como os plasmídeos, podem transportar um ou mais genes de resistência. Muitos dos genes de β-lactamases que conferem resistência as penicilinas e cefalosporinas, estão localizados em tais elementos (BROWN; WRIGHT, 2016).

Os principais mecanismos de resistência aos antimicrobianos incluem alteração do alvo, efluxo, imunidade e destruição catalisada por enzimas. A modificação do alvo pode ocorrer através da mutação dos próprios alvos - por exemplo, as topoisomerases que são o alvo dos antibióticos de fluoroquinolona - ou pela produção de enzimas que modificam alvos antibióticos, como, por exemplo, na metilação ribossômica. O efluxo ocorre através de uma grande família de proteínas que atuam como bombas expulsando os antibióticos do interior da célula. Na resistência à vancomicina uma maquinaria biossintética é responsável pela alteração

da estrutura da parede celular, que tem como objetivo expulsar as drogas da célula. Na imunidade, os antibióticos ou os seus alvos estão ligados por proteínas que impedem a ligação do antibiótico ao seu alvo. Provavelmente, o mecanismo mais específico e evoluído de resistência aos antibióticos são enzimas que reconhecem antibióticos e os modificam de forma a eliminar as características funcionais que lhes permitem interagir com seus alvos. Por exemplo, as β-lactamases que separam hidrolisam o anel β-lactâmico do núcleo que é característico da classe, essencial para a ação antibiótica (BLAIR *et al*., 2015).

A contínua emergência de microrganismos resistentes em instituições de saúde e na comunidade constitui um grande desafio e tem mobilizado órgãos nacionais e internacionais de saúde. No seu primeiro relatório global sobre resistência antimicrobiana, publicado em 2014, a OMS afirmou que o mundo caminha para uma era pós-antibióticos. O relatório discute a resistência de diversos agentes infecciosos, além de descrever estratégias para o combate a resistência aos antimicrobianos, tais como, uso racional dos antibióticos, prescrição adequada e incentivo a pesquisa de novos compostos (WHO, 2014).

A prevalência e a mobilidade de genes de resistência em microrganismos patogênicos previamente sensíveis alcançaram níveis críticos em muitos casos. O desenvolvimento de novos agentes antimicrobianos não tem acompanhado a evolução microbiana. Portanto, considerando a necessidade do desenvolvimento de novos agentes antimicrobianos, a OMS publicou em 2017 uma lista de agentes patogênicos prioritários classificados nos níveis crítico, elevado e médio. O grupo crítico inclui as bactérias multirresistentes dos gêneros *Acinetobacter* e *Pseudomonas*, além de várias bactérias da família *Enterobacteriaceae* (*Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter* spp., *Serratia* spp., *Proteus* spp., *Providencia* spp., *Morganella* spp.). O segundo e terceiro níveis da lista reúnem outras bactérias cada vez mais resistentes aos fármacos, tais como, *S. aureus*, *Salmonella* spp. e *Neisseria gonorrhoeae*. De acordo com a OMS, a publicação do documento é um apelo aos governos para que implementem políticas de incentivo à pesquisa científica básica e avançada, tanto por meio de agências financiadas pelo setor público quanto pelo setor privado (WHO, 2017).

2.2 GÊNERO *Staphylococcus*

O gênero *Staphylococcus* foi descrito pela primeira vez em 1880, pelo cirurgião escocês Alexander Ogston ao observar pus de um abscesso cirúrgico. Em 1884, o médico alemão Friedrich Julius Rosenbach diferenciou duas espécies através da cor de suas colônias: *S. aureus* (do latim *aurum*, ouro) e *S. albus* (do latim *albus*, branco). *S. albus* foi mais tarde renomeado

S. epidermidis por causa da sua ubiquidade na pele humana (LICITRA, 2013). Atualmente, de acordo com a LPSN - *List of Prokaryotic names with Standing in Nomenclature* (2017), o gênero possui 52 espécies e 28 subespécies.

Staphylococcus são cocos Gram-positivos, que se apresentam isolados, aos pares, em cadeias curtas, ou agrupados irregularmente (aspecto semelhante a um cacho de uvas), medem entre 0,5 a 1,5 µm de diâmetro, imóveis, aeróbios ou anaeróbios facultativos, não-esporulados e geralmente não-encapsulados. As colônias medem de 1 a 3 mm de diâmetro e a coloração varia do branco a vários tons de amarelo, dependendo da espécie (MURRAY; ROSENTHAL; PFALLER, 2017).

Staphylococcus estão associados a infecções cutâneas, doenças respiratórias e intoxicação alimentar. As principais espécies envolvidas em processos patogênicos humanos são *S. aureus*, *S. epidermidis* e *S. saprophyticus*. A espécie de maior interesse médico é *S. aureus*, frequentemente relacionada com diversas infecções humanas (LICITRA, 2013).

2.2.1 *Staphylococcus aureus*

Staphylococcus aureus é uma bactéria comensal, frequentemente encontrada nas fossas nasais e na pele de pessoas saudáveis. Entretanto, em situações de imunossupressão ou comprometimento da barreira cutânea por trauma ou cirurgia, *S. aureus* pode causar desde infecções simples, como espinhas e furúnculos, até infecções graves, como meningite e pneumonia. Considerado um dos principais patógenos humanos, *S. aureus* é o principal microrganismo causador de bacteremia, endocardite infecciosa, infecções osteoarticulares, cutâneas, pleuropulmonares e infecções relacionadas a dispositivos médicos, tais como, cateteres intravenosos, válvulas cardíacas, articulações protésicas e tubos endotraqueais (ARYEE; EDGEWORTH, 2016).

S. aureus se destaca por sua elevada frequência e patogenicidade, além de estar associado a múltipla resistência aos antibióticos (ARYEE; EDGEWORTH, 2016). Esse microrganismo conseguiu evoluir adquirindo resistência a quase todos os antibióticos usados para o tratamento das suas infecções. A resistência a penicilina foi relatada em 1942, apenas um ano após a introdução da droga (RAMMELKAMP, 1942). Na década de 1950, as infecções causadas por *S. aureus* resistentes a penicilina ultrapassavam mais da metade dos casos relatados em grandes hospitais. Além disso, o microrganismo foi capaz de desenvolver

resistência a outros antibióticos disponíveis, como eritromicina, estreptomicina, e tetraciclinas (FINLAND, 1955; JESSEN *et al.*, 1969).

A descoberta do mecanismo de resistência a penicilina baseado na produção de β-lactamases, enzimas que promovem a hidrólise do anel β-lactâmico, incentivou o desenvolvimento de antimicrobianos com modificações estruturais que impedissem a atuação de tais enzimas (BONDI; DIETZ, 1945). Portanto, em 1959 foi introduzida a meticilina, uma penicilina semi-sintética resistente as β-lactamases. Contudo, em apenas dois anos surgiram as primeiras cepas de *S. aureus* resistentes a meticilina, identificadas pela sigla MRSA, também resistentes aos demais β-lactâmicos (BRUMFITT; HAMILTON-MILLER, 1989).

As infecções por MRSA foram consideradas um problema predominantemente hospitalar até a década de 1980, quando foram registrados os primeiros casos por cepas de origem comunitária (CA-MRSA, *S. aureus* resistente a meticilina adquirido na comunidade). Desde então, passaram a ser identificadas cepas de MRSA com características genéticas e fenotípicas diferentes das apresentadas pelas cepas hospitalares (HA-MRSA, *S. aureus* resistente a meticilina adquirido em serviços de assistência a saúde) (BASSETTI; NICCO; MIKULSKA, 2009). Infecções por CA-MRSA têm se destacado devido sua rápida emergência, aumento na prevalência e potencial para causar infecções graves (EVANGELISTA; OLIVEIRA, 2015).

Desde o seu surgimento até os dias atuais MRSA constituem um desafio ao tratamento com os antimicrobianos convencionais (STRYJEWSKI; COREY, 2014). As cepas de MRSA evoluem constantemente através da mutação e absorção de elementos genéticos móveis que conferem crescente resistência e virulência. A versatilidade de MRSA está relacionada ao seu potencial de se adaptar ao seu hospedeiro, bem como aos tratamentos desenvolvidos para controlar seus danos invasivos (ARYEE; EDGEWORTH, 2016).

Os principais fatores de virulência (Tabela 1) do *S. aureus* são os componentes da superfície celular, e a síntese de enzimas e de toxinas extracelulares (ZECCONI; SCALI, 2013).

Tabela 1. Principais fatores de virulência de *Staphylococcus aureus* e suas ações no hospedeiro.

Fatores de Virulência		Ações no Hospedeiro
Componentes da superfície celular	Polissacarídeo capsular	Inibe a fagocitose
	Peptidoglicano	Piogênico e quimioatrativo
	Ácido teicóico	Ativa a via alternativa do complemento e estimula a produção de citocinas
	Adesinas	Fixação ao epitélio do hospedeiro
	Proteína A	Interage com a região Fc das IgG (ação antifagocítica)
	Estafiloxantina (Pigmento)	Atua como antioxidante permitindo a desintoxicação de espécies reativas de oxigênio (ROS) geradas pelo sistema imunológico do hospedeiro
Toxinas extracelulares	Hemolisinas	Lisam os eritrócitos
	Leucocidinas	Destroem os leucócitos
	Toxina da Síndrome do Choque Tóxico (TSCT)	Liga-se às moléculas do MHC da classe II e induz a síntese de citocinas causando múltiplas disfunções orgânicas
	Enterotoxinas	Enterotoxigenicidade e efeito emético relacionados à estimulação do sistema nervoso central, após atuação da toxina nos receptores neurais dos intestinos
	Toxina Epidermolítica	Rompe a ligação de células do estrato granuloso da epiderme
Enzimas	Coagulases	Catalisam a conversão de fibrinogênio em fibrina
	Lipases	Hidrolisam os lipídios
	Proteases	Degradam as proteínas
	β- lactamases (Penicilinases)	Quebram o anel β- lactâmico
	Estafiloquinases	Ativam o plasminogênio
	Hialuronidases	Hidrolisam o ácido hialurônico
	Nucleases	Clivam tanto o DNA quanto o RNA

Fonte: ACOSTA *et al.*, 2018; KWIECINSKI *et al.*, 2016; LIU *et al*., 2008; SANTOS *et al*., 2007.

2.3 GÊNERO *Candida*

O gênero *Candida* foi descrito pela primeira vez em 1923, pela micologista alemã Christine Marie Berkhout. As espécies desse gênero são fungos unicelulares, dimórficos, com células esféricas ou ovaladas, produzem brotamentos ou blastoconídios, e podem produzir pseudo-hifas ou hifas verdadeiras, com exceção de *Candida glabrata* (LEDERMANN, 2017).

As espécies de *Candida* residem como comensais, fazendo parte da microbiota normal dos indivíduos sadios. Todavia, quando há uma ruptura no balanço normal da microbiota ou o sistema imune do hospedeiro encontra-se comprometido, as espécies tendem a manifestações agressivas, tornando-se patogênicas. As infecções por *Candida*, denominadas candidíases ou candidoses, envolvem um amplo espectro de doenças oportunistas superficiais e invasivas (VAHEDI *et al*., 2015).

Além disso, as espécies de *Candida* possuem importantes fatores que facilitam a sua multiplicação no hospedeiro possibilitando a produção de infecções. Estes fatores estão relacionados principalmente a adesão ao tecido do hospedeiro, capacidade de transformação de leveduras para hifas, variabilidade de mudança fenotípica (fenômeno switching), produção de exoenzimas hidrolíticas e hemolisinas (SOLIS *et al*., 2018).

O principal agente causador das candidíases é *C. albicans*, estando presente em muitos dos isolados de amostras clínicas, em seguida estão *C. parapsilosis*, *C. glabrata*, *C. tropicalis* e *C. krusei* (VALLABHANENI *et al*., 2016).

2.3.1 *Candida glabrata*

Diferente das outras espécies do gênero *Candida*, *C. glabrata* não é dimórfica, consequentemente é encontrada na forma de blastoconídios sob todas as condições ambientais, sendo a única espécie do gênero que não forma pseudo-hifas. Outra característica distintiva de *C. glabrata* é o seu genoma haplóide, em comparação com o genoma diplóide das outras espécies do gênero (BOLOTIN-FUKUHARA; FAIRHEAD, 2016). Em meio Ágar Sabouraud Dextrose (ASD), forma colônias pequenas, homogêneas e brilhantes, que são relativamente indistinguíveis das outras espécies de *Candida* (BARBEDO; SGARBI, 2010).

Historicamente, *Candida glabrata* era considerado um microrganismo saprófito e com baixo poder patogênico, raramente causando infecções graves em humanos. No entanto, após o uso generalizado e aumentado da terapia imunossupressora juntamente com a terapia antimicótica de amplo espectro, a frequência de infecções mucosas e sistêmicas causadas por

C. glabrata aumentou significativamente. O patógeno está entre os três principais agentes causadores de candidíase (CAVALHEIRO *et al*., 2019).

C. glabrata desenvolveu estratégias como adesão, formação de biofilmes, capacidade de sobrevivência nos macrófagos e produção de pigmentos, que justificam características como a sua elevada resistência aos fármacos e capacidade para sobreviver em condições escassas de nutrientes. Tais características permitem que *C. glabrata* seja considerado não apenas um microrganismo comensal, mas também um patógeno oportunista de sucesso (MIYAZAKI; KOHNO, 2014).

A adesão constitui um mecanismo essencial para a colonização inicial e persistente do microrganismo. *C. glabrata* apresenta um vasto grupo de proteínas glicosilfosfatidilinositol (GPI) ancoradas na parede celular, muitas delas são potenciais adesinas (MIYAZAKI; KOHNO, 2014). Além disso, *C. glabrata* possui a capacidade de colonizar os tecidos do hospedeiro assim como superfícies abióticas onde desenvolve complexas estruturas de biofilme. Os biofilmes representam uma das estratégias de resistência de *C. glabrata*, pois diminuem a sua sensibilidade a diversos antifúngicos e originam infecções persistentes (RODRIGUES *et al*., 2017).

Após a adesão, o passo seguinte na patogênese de *Candida* é a invasão. Infecções por *C. albicans* são caracterizadas por forte infiltração de neutrófilos e a sua flexibilidade morfológica entre levedura e forma filamentosa desempenha um papel importante na fuga à fagocitose e na penetração no tecido hospedeiro. De um modo geral, a presença de hifas e os danos celulares causados no hospedeiro em infecções por *C. albicans* origina uma resposta pró-inflamatória por meio de citocinas muito superior à causada por *C. glabrata*. *C. glabrata*, contudo, não produz hifas e o seu comportamento sutil permite a sua persistência no hospedeiro sem causar nenhuma resposta imunitária relevante (BRUNKE; HUBE, 2013).

C. glabrata era considerada uma espécie não pigmentada, contudo alguns estudos comprovaram a produção de pigmentos derivados do indol. A produção destes depende da presença de triptofano como fonte de nitrogênio. A principal função da produção de pigmentos é a proteção do patógeno, sendo por isso considerado como um fator de virulência. Além disso, os pigmentos podem ter várias funções biológicas incluindo efeitos antioxidantes, que contrariam o efeito de espécies reativas de oxigênio (ROS) produzidas pelo sistema imunitário do hospedeiro (RODRIGUES; SILVA; HENRIQUES, 2014).

As infecções por *C. glabrata* podem ser mucosas ou sistêmicas, possuem alta taxa de mortalidade, e são consideradas infecções de tratamento difícil, em muitos casos resistentes à diversos agentes antifúngicos azóis, especialmente o fluconazol. Em geral, isolados clínicos de *C. glabrata* apresentam menor sensibilidade ao fluconazol, e como consequência, um aumento nos índices de infecção por *C. glabrata* tem sido observado em pacientes com exposição prolongada ao fluconazol. Além dos problemas terapêuticos associados aos azóis, estudos demonstram uma menor sensibilidade dos isolados clínicos também para anfotericina B. Portanto, infecções por *C. glabrata* também são mais frequentes em pacientes previamente expostos a anfotericina B (ALANGADEN, 2011).

Tabela 2. Principais fatores de virulência de *Candida glabrata* e suas ações no hospedeiro.

Fatores de Virulência		Ações no Hospedeiro
Componentes da superfície celular	Adesinas	Fixação ao epitélio do hospedeiro
Toxinas extracelulares	Hemolisinas	Lisam os eritrócitos
Enzimas	Proteinases	Auxiliam na invasão tecidual através da sua atividade proteolítica
	Fosfolipases	Auxiliam na invasão tecidual através da hidrólise dos fosfolipídios
	Coagulases	Catalisam a conversão de fibrinogênio em fibrina

Fonte: DAS MOHAN; BALLAL, 2008; DEORUKHKAR; SAINI; MATHEW, 2014.

2.4 PLANTAS MEDICINAIS

Desde a antiguidade os recursos vegetais são utilizados para o tratamento de diversas doenças. Os primeiros registros escritos sobre a utilização medicinal das plantas datam mais de 5.000 anos e foram realizados pela antiga civilização Suméria, que anotaram seus conhecimentos sobre cerca de 250 espécies vegetais em placas de argila (SALEHI *et al*., 2018).

Por volta de 2.500 a.C., o imperador chinês Shen Nung, considerado o fundador da fitoterapia chinesa, escreveu o livro “Shen Nung Pen Tsao Ching”. Neste livro estão listadas 365 plantas medicinais e seus usos. Em 1.500 a.C., os antigos egípcios escreveram o Papiro Ebers, que contém informações sobre aproximadamente 850 plantas medicinais

(PETROVSKA, 2012). Deste modo, as propriedades curativas das plantas foram sendo observadas, registradas e transmitidas para as gerações sucessivas ao longo do tempo.

No âmbito da cultura ocidental, a civilização Grega contribuiu de maneira significativa para o conhecimento das plantas medicinais. Hipócrates (460-361 a.C.) catalogou centenas de drogas vegetais. Teofrasto (372-287 a.C.), inicialmente e, Dioscorides (37-90 d.C.), mais tarde, foram autores de um valioso conjunto de volumes sobre plantas usadas na cura de doenças. O trabalho desenvolvido pelo médico grego Pedanius Dioscorides, deu origem a famosa enciclopédia e farmacopeia "De Materia Medica", publicada no primeiro século após Cristo, na qual as características e propriedades de 600 plantas são descritas (CORRÊA; BATISTA; QUINTAS, 2011).

Durante o Renascimento (período entre meados do século XIV e final do século XVI), o empirismo da medicina e da farmácia medievais, cedem lugar a experimentação. O estudo das plantas entra no período científico ao adotar a classificação e a descrição taxonômica das espécies (CUNHA; RIBEIRO; ROQUE, 2009). No século XIX, químicos e farmacêuticos empenharam-se no isolamento de constituintes ativos a partir de produtos vegetais. Portanto, iniciou-se uma nova fase na utilização científica das plantas, com a substituição destas e dos seus extratos, pelos compostos responsáveis pela sua ação farmacológica (DA CRUZ MONTEIRO; BRANDELLI, 2017).

Um dos primeiros exemplos desta abordagem foi o desenvolvimento do ácido acetilsalicílico a partir da salicina obtida da casca do salgueiro, uma espécie vegetal pertencente ao gênero *Salix* L. (SALICACEAE). Assim como, a utilização dos glicosídeos digitoxina e digoxina, de plantas do gênero *Digitalis* L. (SCROPHULARIACEAE), no tratamento da fibrilação atrial; da reserpina, proveniente da espécie *Rauvolfia serpentina* Benth. ex Kurz (APOCYNACEAE), como anti-hipertensiva; da pilocarpina, oriunda de *Pilocarpus jaborandi* Holmes (RUTACEAE), no tratamento do glaucoma; dos alcalóides vincristina e vimblastina, oriundos da *Catharanthus roseus* (L.) G. Don (APOCYNACEAE), por sua atividade antineoplásica, na leucemia infantil e na doença de Hodkin's; da efedrina, a partir da *Ephedra sinica* Stapf. (EPHEDRACEAE), como anti-histamínico; e da quinina, extraída da casca de espécies de *Cinchona* L. (RUBIACEAE), como antimalárica (DA CRUZ MONTEIRO; BRANDELLI, 2017).

A partir do início do século XX, estudos que correlacionam a estrutura química dos compostos ativos obtidos de plantas com a sua respectiva ação fisiológica têm permitido a

descoberta de novas moléculas naturais de elevada atividade farmacológica. O desenvolvimento da química analítica através dos métodos cromatográficos e espectrométricos, além do advento de equipamentos cada vez mais sofisticados para essas análises (KATERYNA, 2017).

As plantas medicinais são importantes para a pesquisa farmacológica e no desenvolvimento de drogas, não somente quando seus constituintes são usados diretamente como agentes terapêuticos, mas também como matérias-primas para a síntese, ou modelos para compostos farmacologicamente ativos. A OMS (Organização Mundial de Saúde) define planta medicinal como sendo "todo e qualquer vegetal que possui, em um ou mais órgãos, substâncias que podem ser utilizadas com fins terapêuticos ou que sejam precursores de fármacos semi-sintéticos" (VAN; WINK, 2018).

O Brasil é o país de maior biodiversidade do planeta. Entre os elementos que compõem a biodiversidade, as plantas são a matéria-prima para a fabricação de fitoterápicos e outros medicamentos. Além de seu uso como substrato para a fabricação de medicamentos, as plantas são também utilizadas em práticas populares e tradicionais como remédios caseiros e comunitários, processo conhecido como medicina tradicional. Além desse acervo genético, o Brasil é detentor de rica diversidade cultural e étnica que resultou em um acúmulo considerável de conhecimentos tradicionais, passados de geração a geração, entre os quais se destaca o vasto acervo de conhecimentos sobre manejo e uso de plantas medicinais (BRASIL, 2016).

Na perspectiva de integralidade da atenção e promoção a saúde, o Ministério da Saúde aprovou, no ano de 2005, o uso terapêutico de plantas pelo Conselho Nacional de Saúde, da Política Nacional de Práticas Integrativas e Complementares (PNPIC) no Sistema Único de Saúde (SUS), e em 2006 publicou a "Política Nacional de Plantas Medicinais e Fitoterápicos". Tais medidas tiveram como principal objetivo a ampliação das opções terapêuticas aos usuários do SUS, garantindo o acesso as plantas medicinais, fitoterápicos e serviços relacionados a fitoterapia, com segurança, eficácia e qualidade (BRASIL, 2016).

Os fitoterápicos constituem uma importante fonte de inovação em saúde, sendo objeto de interesses empresariais privados e fator de competitividade do Complexo Produtivo da Saúde. Esse contexto impõe a necessidade de uma ação transversal voltada ao fortalecimento da base produtiva e de inovação local e à competitividade da indústria nacional. O desenvolvimento do setor de plantas medicinais e fitoterápicos pode se configurar como importante estratégia para o enfrentamento das desigualdades regionais existentes no Brasil,

podendo prover a necessária oportunidade de inserção socioeconômica das populações de territórios caracterizados pelo baixo dinamismo econômico e indicadores sociais precários (BRASIL, 2016).

2.5 METABOLISMO VEGETAL

Os constituintes químicos encontrados no Reino Vegetal são sintetizados e degradados por inúmeras reações anabólicas e catabólicas. Os produtos resultantes do metabolismo vegetal estão divididos em dois grandes grupos: o dos metabólitos primários ou macromoléculas, provenientes dos processos fotossintéticos e com funções vitais bem definidas; e o dos metabólitos secundários ou micromoléculas, originados à custa de energia, através de rotas biossintéticas diversas e, frequentemente desconhecidas (KOPRIVA, 2015).

Os metabólitos primários são representados pelos açúcares, aminoácidos, ácidos graxos, nucleotídeos e seus polímeros derivados. Os metabólitos secundários podem ser divididos em três grandes grupos: terpenos (formados por unidades isoprênicas de cinco carbonos, através da via do acetato), compostos fenólicos (formados a partir de um anel aromático com um ou mais substituintes hidroxílicos, através da via ácido chiquímico), e alcalóides (formados principalmente a partir de aminoácidos aromáticos) (WIERMANN, 2013; RAVEN; EVERT; EICHHORN, 2014).

No passado, para alguns autores, os metabólitos secundários nada mais eram do que subprodutos do metabolismo primário, considerados desperdício fisiológico ou produtos de desintoxicação celular. Entretanto, o fato do vegetal utilizar rotas biossintéticas elaboradas, com elevados gastos energéticos, conduziu à hipótese de que as plantas consomem tamanha energia com a finalidade de sintetizar compostos necessários para a sua sobrevivência, preservação e reprodução. O estudo do metabolismo secundário abrange a fisiologia e a bioquímica vegetal, bem como aspectos ecológicos e evolutivos (ZHAO *et al*., 2013).

2.5.1 Metabolismo Secundário de Plantas

Diferente das vias que dão origem aos produtos do metabolismo primário, as quais sintetizam, degradam e convertem compostos comumente encontrados em todos os seres vivos, existem vias metabólicas direcionadas para a produção de constituintes que têm uma distribuição mais limitada na natureza. Tais compostos, chamados de metabólitos secundários, são encontrados apenas em organismos específicos e são uma expressão da individualidade das espécies (KOPRIVA, 2015).

As rotas metabólicas que os originam os metabólitos secundários, geralmente, são ativadas durante alguns estágios particulares do crescimento e desenvolvimento, em períodos de estresse causados por limitações nutricionais e pelo ataque de herbívoros ou microrganismos patogênicos. Deste modo, os metabólitos secundários asseguram a sobrevivência da espécie vegetal em seu ecossistema, desempenhando importantes papéis ecológicos, como atração de polinizadores, adaptação ao estresse ambiental e defesa química. Além disso, a composição química e a concentração destes compostos podem variar a depender dos fatores ambientais e ecológicos, das condições edafoclimáticas, da sazonalidade e até mesmo do ritmo circadiano (RAVEN; EVERT; EICHHORN, 2014).

Os terpenos estão presentes em grandes quantidades nos óleos essenciais, resinas e ceras, e são comercialmente importantes devido a sua ampla utilização na indústria como flavorizantes, fármacos, perfumes e inseticidas. Os compostos fenólicos constituem um grupo heterogêneo de componentes químicos, como flavonóides, taninos e cumarinas. Os alcalóides possuem nitrogênio, geralmente na forma amina, em sua estrutura. Neste grupo destacam-se os glicosídeos cianogênicos, considerados alcalóides de defesa anti-herbívoros (TAIZ; ZEIGER, 2013).

2.6 Óleos Essenciais

Manuscritos antigos descrevem a utilização de óleos essenciais (OEs) por diversas civilizações, tais como, Grega, Persa e Egípcia. Os primeiros relatos datam mais de 2.000 a.C., e evidenciam a sua utilização no embalsamamento de cadáveres, em rituais religiosos e como fragrâncias. Séculos depois começaram a surgir as primeiras descrições sobre as propriedades terapêuticas dos OEs (BRITO *et al*., 2013).

Os OEs são compostos voláteis complexos constituídos por uma mistura de substâncias resultantes do metabolismo secundário de plantas medicinais e aromáticas (ALI *et al*., 2015). Conforme a ISO (International Organization for Standardization) (1997), OEs são definidos como produtos obtidos de partes de plantas através da destilação por arraste de vapor d'água ou prensagem dos pericarpos de frutos cítricos. Deste modo, a definição dos OEs não se limita somente a sua composição química, considerando também o processo utilizado para sua obtenção.

Segundo CASSEL *et al*. (2009), dependendo do método de extração utilizado, a composição química do óleo pode variar significativamente. Existem diferentes métodos de

extração, tais como, destilação por arraste de vapor d'água, prensagem, enfloração (*enfleurage*) e extração por dióxido de carbono (CO_2) supercrítico. Para a escolha do melhor método, deve-se levar em consideração a localização do óleo na planta e a finalidade do seu uso (BAKKALI *et al*., 2008).

Existem três variações no processo de destilação com vapor d'água: destilação com água (hidrodestilação), destilação com água e vapor, e destilação a vapor (ROSTAGNO; PRADO, 2013). Na hidrodestilação, a planta aromática permanece em contato com a água fervente, podendo estar completamente imersa ou flutuando. Esta técnica é realizada frequentemente em laboratórios utilizando-se o aparelho Clevenger. Na destilação com água e vapor, o material a ser destilado é apoiado sobre uma placa perfurada ou inserido em uma rede e colocado logo acima do fundo do destilador. E na destilação a vapor, a água não é mantida na parte inferior do destilador, e o vapor é introduzido através de serpentinas (BUSATO *et al*., 2014; EL ASBAHANI *et al*., 2015).

A enfloração ou *enfleurage* é utilizada principalmente pelas indústrias de perfumes para a extração dos OEs de pétalas de flores que apresentam baixo rendimento e alto valor comercial (BAKKALI *et al*., 2008). A técnica consiste em colocar as pétalas sobre uma placa de vidro com uma camada de gordura, a temperatura ambiente, durante período suficiente para absorção total do óleo pela gordura. As pétalas vão sendo trocadas até que a gordura fique saturada e seja, posteriormente, tratada com álcool. Para a retirada do álcool, é feita uma destilação a baixa temperatura (EL ASBAHANI *et al*., 2015).

A extração por CO_2 supercrítico é considerado um método bastante eficiente (BAKKALI *et al*., 2008). A técnica consiste em liquefazer o CO_2 por compressão, seguido de um aquecimento a 31 ºC, desta forma, o CO_2 atinge um estado intermediário com viscosidade de um gás e capacidade de dissolução elevada, como de um líquido. Terminada a extração, o CO_2 volta à temperatura ambiente, sendo totalmente eliminado do óleo obtido. Nenhum traço de solvente é encontrado no óleo obtido por esse método, além disso, por não utilizar elevadas temperaturas, não há o risco de ocorrer a degradação nem a formação de novos compostos (EL ASBAHANI *et al*., 2015).

Nas angiospermas dicotiledôneas encontram-se o maior número de famílias botânicas com espécies produtoras de OEs, com destaque para Apiaceae, Lauraceae, Lamiaceae, Myrtaceae, Oleaceae, Rosaceae e Rutaceae (BANDONI; CZEPACK, 2008; FIGUEIREDO *et al*., 2008). Os OEs podem estar presentes em diferentes órgãos da espécie vegetal, como folhas,

flores, frutos, ramos, caules e raízes. Após sua biossíntese, são armazenados em estruturas específicas tais como, tricomas glandulares, células parenquimatosas diferenciadas e canais oleíferos (BASER; BUCHBAUER, 2015).

A composição química dos OEs é complexa, cerca de 20 a 60 componentes bioativos são observados em sua maioria. Além disso, a caracterização química comumente revela a presença de 2 ou 3 componentes majoritários presentes em elevada concentração (20-70%). Seus constituintes químicos podem ser divididos em duas classes, conforme a origem biossintética: aromática e terpênica, sendo mais frequentes os compostos provenientes da classe terpênica (PANDEY; SINGH; TRIPATHI, 2014).

Os compostos da classe aromática são constituídos por uma cadeia lateral de três átomos de carbono derivados de aminoácidos aromáticos, oriundos da via do ácido chiquímico. Nesta via, a partir do ácido chiquímico, é produzido o aminoácido fenilalanina que, por ação da enzima fenilalanina amônia-liase (PAL) forma o ácido cinâmico e o ácido *p*-cumárico. A redução da cadeia lateral destes ácidos leva à formação de alilbenzenos e propenilbenzenos, esqueletos carbônicos dos fenilpropanóides (BASER; BUCHBAUER, 2015).

Os compostos da classe terpênica são sintetizados pela condensação de unidades pentacarbonadas, o difosfato de isopentenila (IPP) e seu isômero difosfato de dimetilalila (DMAPP). A formação do IPP pode ocorrer por duas rotas biossintéticas: a via clássica ou via do mevalonato (MEV), responsável pela formação dos sesquiterpenos e triterpenos, que ocorre preferencialmente no citosol e cujos precursores são piruvato e acetilcoenzima A; e a via alternativa ou via do metileritritol-fosfato (MEP), que origina os monoterpenos, diterpenos e tetraterpenos, e ocorre preferencialmente nos plastídeos e tem como precursores piruvato e gliceraldeído-3-fosfato (MEWALAL *et al*., 2017).

Após a síntese do IPP, este é convertido em seu isômero DMAPP, através da enzima IPP isomerase. A junção de uma molécula de IPP a uma molécula de DMAPP forma o difosfato de geranila (GPP), precursor dos monoterpenos (C_{10}). À medida que são adicionadas unidades de IPP, formam-se o difosfato de farnesila (FPP), precursor dos sesquiterpenos (C_{15}), e o difosfato de geranil geranila (GGPP), precursor dos diterpenos (C_{20}). Os triterpenos (C_{30}) são formados pela junção de duas unidades FPP e os tetraterpenos (C_{40}) pela junção de duas unidades GGPP (MEWALAL *et al*., 2017).

Monoterpenos e sesquiterpenos são os derivados terpênicos mais comuns nos OEs. Os monoterpenos são altamente voláteis, e podem ser hidrocarbonetos (limoneno, α-pineno, *p*-cimeno) ou compostos oxigenados, com funções álcool (linalol, mentol, geraniol), aldeído (citral, citronelal), cetona (pinocarvona, fenchona, mentona), éter (mentofurano, eucaliptol) ou éster (acetato de linalila, acetato de isobornila). Além disso, os monoterpenos são os principais componentes de OEs bioativos. Os sesquiterpenos, por serem cadeias carbônicas maiores, são menos voláteis que os monoterpenos. E assim como estes, podem ser hidrocarbonetos (β-cariofileno, farneseno) ou oxigenados, com funções álcool (β-santalol, bisabolol, viridiflorol), cetona (β-vetivenona, germacrona) ou epóxido (óxido de cariofileno) (BAKKALI *et al.*, 2008).

A composição química dos OEs pode variar em função de fatores ambientais como a umidade do ar, disponibilidade hídrica, condições de solo, intensidade luminosa, temperatura e herbivoria. Fatores endógenos como idade da espécie vegetal e estádio fenológico, bem como a existência de quimiotipos também podem acarretar mudanças na composição e no rendimento dos OEs (BASER; BUCHBAUER, 2015).

Os OEs exercem funções importantes e específicas dentro das plantas, sendo considerados compostos vitais para a manutenção da espécie. Atuam na defesa das plantas contra os seus inimigos naturais, como os herbívoros, e contra microrganismos patogênicos, como fungos e bactérias (BAKKALI *et al*., 2008). Podem atuar também na proteção de folhas e meristemas contra perda de água e aumento da temperatura, auxiliando na termotolerância da planta ao estresse abiótico (GLINWOOD; NINKOVIC; PETTERSSON, 2011). Podem também estar envolvidos nos processos de atração e orientação de polinizadores, e de dispersão de sementes. Além disso, apresentam ainda efeitos alelopáticos (ARORA *et al*., 2017).

Além da importância ecológica nas relações da planta com o ambiente, com os animais e com outras plantas, os OEs constituem um grupo de substâncias cada vez mais estudado por sua importância econômica. Diversas propriedades farmacológicas são atribuídas a estes compostos, tais como, antimicrobiana (PRAKASH *et al*., 2018), antioxidante (STANOJEVIĆ *et al*., 2018), anti-inflamatória (SIENIAWSKA *et al*., 2019), antitumoral (HAIYAN *et al*., 2016), anestésica (CAN *et al*., 2018), repelente/inseticida (ARENA *et al*., 2017), acaricida (FERREIRA *et al*., 2018) e larvicida (PAVELA; GOVINDARAJAN, 2017).

A complexidade química dos OEs dificulta a análise dos seus componentes bioativos. Em muitos casos registrados na literatura, o constituinte majoritário é responsável pela

atividade biológica. No entanto, esta pode ser atribuída à ação sinérgica ou antagônica de vários dos seus componentes (BAKKALI *et al.,* 2008).

2.6.1 Propriedades Antimicrobianas dos Óleos Essenciais

A atividade antimicrobiana de OEs e seus componentes químicos têm sido relatada há muito tempo. As propriedades antimicrobianas dos OEs dependem principalmente da sua constituição química. A presença de diferentes tipos de terpenos, fenóis, aldeídos e outros compostos antimicrobianos justifica a ação diversificada dos óleos essenciais contra agentes patogênicos bacterianos, fúngicos e virais (AKHTAR; DEGAGA; AZAM, 2014).

O efeito da atividade antibacteriana de óleos essenciais pode inibir o crescimento de bactérias (bacteriostático) ou destruir células bacterianas (bactericida). A eficácia dos óleos essenciais difere de um tipo para outro, bem como contra diferentes bactérias alvo, dependendo da sua estrutura (bactérias Gram-positivas e Gram-negativas). Por exemplo, os óleos de sândalo (*Santalum album*) e vetiver (*Vetiveria zizanioides*) exibem maior atividade inibitória contra bactérias Gram-positivas; no entanto, eles não conseguem inibir cepas bacterianas Gram-negativas (HAMMER; CARSON, 2011; RAUT; KARUPPAYIL,2014).

Em geral, o mecanismo de ação antibacteriana é mediado por uma série de reações bioquímicas na célula bacteriana, que são dependentes do tipo de constituintes químicos presentes no OE (NAZZARO *et al*., 2013). A principal atuação dos OEs consiste em desestabilizar a arquitetura celular bacteriana, levando à degradação da integridade da membrana e ao aumento da permeabilidade, o que interrompe muitas atividades celulares, incluindo a produção de energia, o transporte e outras funções metabólicas reguladoras. Devido à sua natureza lipofílica, os OEs são facilmente penetráveis através das membranas celulares bacterianas. O aumento da permeabilidade da membrana celular bacteriana, leva ao vazamento de componentes celulares e perda de íons (SAAD; MULLER; LOBSTEIN, 2013; RAUT; KARUPPAYIL, 2014).

O efeito antibacteriano dos OEs também está ligado à potenciais de membrana reduzidos, à interrupção das bombas de prótons e à depleção do ATP. Essa alteração na organização celular pode causar um efeito em cascata, resultando em outras organelas celulares afetadas, como por exemplo as mitocôndrias. Os óleos essenciais passam pela parede celular e membrana citoplasmática, o que pode perturbar o arranjo de ácidos graxos, fosfolípidos e polissacarídeos (SAAD; MULLER; LOBSTEIN, 2013). Esses eventos podem ser responsáveis

pela coagulação de componentes celulares internos no citoplasma e quebra das ligações entre as camadas de lipídios e proteínas. Além disso, alguns OEs podem inibir a rede de Quorum Sensing de comunicação célula-célula mediada por várias moléculas sinalizadoras bacterianas (SZABÓ *et al*., 2010).

As ações antifúngicas dos óleos essenciais são semelhantes às dos mecanismos antibacterianos. Geralmente, a exposição de óleos essenciais leva à coagulação dos componentes celulares devido ao dano irreversível da membrana celular. Nas células de levedura, os óleos essenciais estabelecem um potencial de membrana através da membrana celular e perturbam a produção de ATP, o que leva ao dano da membrana celular (ALEKSIC; KNEZEVIC, 2014). A permeabilização da membrana citoplasmática, causa alterações no fluxo de eletrons da cadeia transportadora de eletrons (CTE), levando à desintegração das membranas mitocondriais. Os óleos essenciais também podem perturbar a despolarização das membranas mitocondriais afetando os canais de íons, especialmente os íons Ca2 +, as bombas de protões e os pools de ATP e, portanto, diminuem o potencial da membrana. Essa alteração na fluidez das membranas pode causar vazamento eletrolítico e dificultar as vias do citocromo C, o metabolismo das proteínas e as concentrações de íons cálcio. Portanto, a permeabilização das membranas mitocondriais internas e externas pode resultar na apoptose celular ou necrose levando à morte celular.

A avaliação dos óleos essenciais no combate a microrganismos patogênicos multirresistentes demonstra o seu elevado potencial para o desenvolvimento de novos antimicrobianos (SWAMY; AKHTAR; SINNIAH, 2016).

2.7 FAMÍLIA MYRTACEAE

A família Myrtaceae, pertencente à ordem Myrtales, compreende cerca de 132 gêneros com aproximadamente 5.950 espécies. Dividida em duas subfamílias, Myrtoideae e Psiloxyloideae, a família ocorre principalmente no Hemisfério Sul, com destaque para a Austrália (cerca de 85 gêneros) e a América neotropical, especialmente no Brasil, onde são encontrados 23 gêneros e mais de 1.000 espécies (BIDA *et al*., 2018).

As espécies desta família são arbóreas ou arbustivas, geralmente perenifólias e aromáticas, muitas ricas em óleos essenciais utilizados pelas indústrias alimentícia, farmacêutica e de perfumaria. Caracterizam-se por apresentar glândulas oleíferas translúcidas

presentes nas folhas, flores e frutos, o que justifica a produção relativamente alta de óleos essenciais pelos seus representantes (WATSON; DALLWITZ, 2017).

A família inclui uma grande variedade de plantas cultivadas para fins econômicos e ornamentais, tais como, os eucaliptos (gênero *Eucalyptus*), o cravo-da-índia (*Syzygium aromaticum*), o calistemo (*Callistemon viminalis*), a pitangueira (*Eugenia unifora*), a goiabeira (*Psidium guajava*) e a jabuticabeira (*Plinia cauliflora*) (LIU *et al*., 2018). Espécies como o araçazeiro (*Psidium cattleyanum*), a guabirobeira (*Campomanesia xanthocarpa*) e a uvaieira (*Eugenia pyriformis*) são também usadas na medicina popular e cultivadas em hortas domésticas (PEREIRA *et al*., 2012). No Brasil, diversas espécies de Myrtaceae são empregadas nos casos de distúrbios gastrointestinais, estados hemorrágicos e nas doenças infecciosas. As partes mais usadas das plantas são folhas, cascas e frutos (CRUZ; KAPLAN, 2012).

Myrtaceae são frequentemente usadas na medicina popular; portanto, muitas espécies foram testadas para atividades biológicas. Diversas espécies exibiram propriedades antimicrobianas e parece que a bioatividade está associada à presença de compostos fenólicos ou a um alto nível de terpenos oxigenados. Óleos essenciais e extratos de várias espécies são capazes de inibir o crescimento de microrganismos relacionados às doenças de pele, cárie dentária e deterioração de alimentos. Os compostos fenólicos (ácidos fenólicos, polifenóis e flavonóides) nas ervas e especiarias, juntamente com os óleos essenciais, atraem cada vez mais atenção devido às atividades antioxidantes e ao uso como agentes aromatizantes. Os óleos essenciais de algumas espécies também demonstraram atividades citotóxicas, antioxidantes, antilarvas, antinociceptivas, anti-inflamatórias e analgésicas. Os resultados desses estudos evidenciam o grande potencial da família Myrtaceae na descoberta de novos fármacos (STEFANELLO; PASCOAL; SALVADOR, 2011).

2.8 GÊNERO *Eugenia*

O gênero *Eugenia* é considerado um dos maiores da família Myrtaceae, compreende aproximadamente 1.100 espécies, das quais cerca de 400 distribuem-se no Brasil. As espécies do gênero estão distribuídas principalmente em regiões tropicais e subtropicais da América, em regiões tropicais da Ásia e algumas espécies também ocorrem na Austrália e na África. O gênero encontra-se bem representado no Brasil, considerando não apenas a abundância e frequência de suas espécies, mas também a riqueza específica das espécies que ocorrem no país (CANTANHEDE-FILHO *et al*., 2017).

Muitas das espécies do gênero são cultivadas como plantas ornamentais, e além disso, algumas espécies produzem frutos comestíveis (SARDI *et al*., 2017). A cerejeira (*Eugenia involucrata* DC.) e a pitangueira (*Eugenia uniflora* L.) são exemplos de espécies apreciadas gastronomicamente por produzirem frutos que são consumidos *in natura* ou processados.

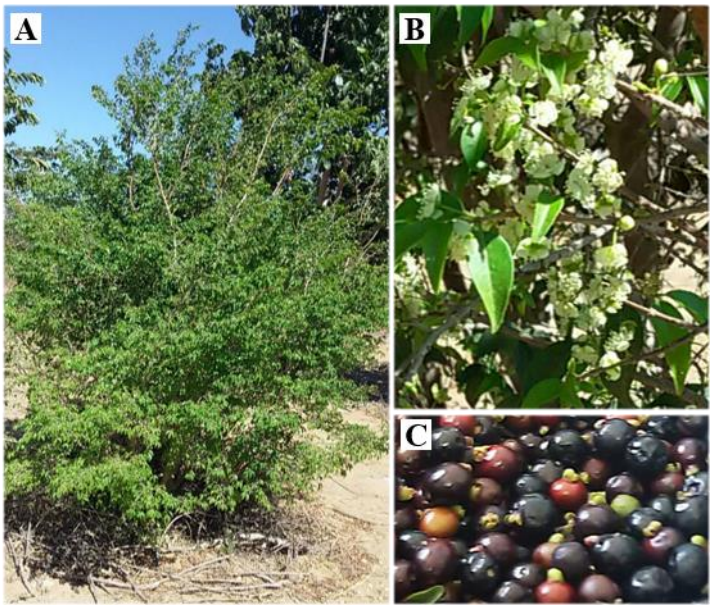
Estudos fitoquímicos em espécies do gênero frequentemente relatam a presença de metabólitos secundários como flavonoides, triterpenoides, taninos e ácidos fenólicos (GUPTA *et al*., 2014; HAMINIUK *et al*., 2014; TEIXEIRA *et al*., 2015). Muitos destes compostos possuem propriedades biológicas de interesse farmacológico, como por exemplo, os flavonoides, que apresentam atividades antioxidante, anti-inflamatória e antitumoral (VEZZA *et al*., 2016).

O gênero *Eugenia*, assim como outros da família Myrtaceae, é conhecido pela produção de óleos essenciais com propriedades aromáticas e medicinais. Entretanto, os óleos do gênero destacam-se por apresentar uma grande diversidade química, com aproximadamente 300 compostos identificados nas espécies analisadas. Sesquiterpenos cíclicos e monoterpenos são os compostos mais abundantes encontrados nestes óleos, com algumas espécies produzindo compostos aromáticos alifáticos em grande quantidade (BIDA *et al*., 2018).

2.8.1 *Eugenia gracillima* Kiaersk.

Descrita por Hjalmar Kiaerskou em 1893, *Eugenia gracillima* Kiaersk. (Myrtaceae) (Figura 1) é uma espécie nativa e endêmica do Brasil. De acordo com a Flora do Brasil (2017), a espécie distribui-se geograficamente no País nas regiões Nordeste (Bahia, Ceará e Pernambuco), Centro-Oeste (Goiás, Mato Grosso do Sul, Mato Grosso), Sudeste (São Paulo) e Sul (Paraná, Rio Grande do Sul, Santa Catarina). Possui como sinônimos *Eugenia multipunctata* Mattos & D. Legrand, *Eugenia neomultipunctata* Sobral, *Eugenia leptomischa* Kiaersk., e *Eugenia klappenbachiana* Mattos & D. Legrand.

Figura 1. *Eugenia gracillima* Kiaersk.: (A) Arbusto, (B) flores e (C) frutos.



Fonte: O autor, 2017.

A planta é um arbusto variando em altura de 1,5 a 5,0 metros. Conhecida popularmente como murta na região nordeste do Brasil ou como cambuí em outras regiões. Seus frutos são consumidos frescos ou processados em forma de polpa, suco, géleia, licor ou sobremesas. No nordeste brasileiro, os frutos da espécie são socioeconomicamente importantes (ARAUJO *et al*., 2016).

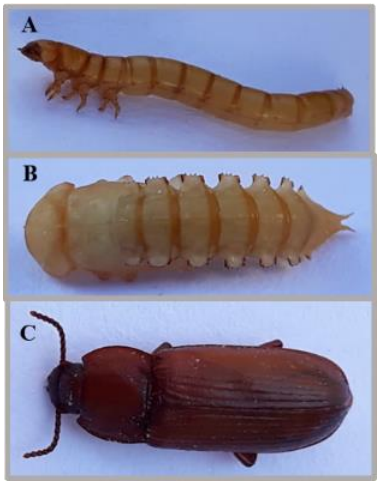
Um estudo avaliou a composição química e as atividades antioxidante e antiprotozoária do óleo essencial de *E. gracillima* (EgEO). Sesquiterpenos hidrocarbonetos (91,22%), sesquiterpenos oxigenados (7,45%) e monoterpenos (1,01%) foram as principais classes de compostos químicos identificadas. Os constituintes voláteis mais abundantes do EgEO foram germacreno D (16,10%), c-muuroleno (15,60%), biciclogermacreno (8,53%), germacreno B (7,43%) e D-elemeno (6,06%). O óleo mostrou atividade antioxidante fraca a moderada. O EgEO foi altamente seletivo para as promastigotas de *Leishmania braziliensis* e *Leishmania infantum* com índices seletivos de 73,66 e 71,41, respectivamente. O EgEO não inibiu o *Trypanosoma cruzi* (SAMPAIO *et al.*, 2019).

2.9 *Tenebrio molitor*

Tenebrio molitor (Linnaeus, 1758) (Figura 2), também conhecido como larva de farinha, pertence a ordem Coleoptera e a família Tenebrionidae. Este inseto exibe holometabolismo (metamorfose completa), no qual seu ciclo de vida compreende quatro fases distintas que são:

ovo, larva, pupa e imago. A fase larval apresenta uma série de estágios ou instares, que é alcançada após o processo de ecdise (PARK *et al*., 2014).

Figura 2. Fases do ciclo de vida de *Tenebrio molitor* L.: (A) larva, (B) pupa e (C) adulto.



Fonte: O autor, 2018.

Em geral, o ciclo de vida de *T. molitor* possui aproximadamente quatro a cinco meses de duração, podendo ainda se estender até doze meses dependendo das condições climáticas, em especial a temperatura do ambiente (BJØRGE *et al*., 2018). Estudos demonstraram que o número de instares de *T. molitor* varia em resposta a diferentes fatores, como temperatura, umidade, concentração de oxigênio, qualidade de nutrientes e densidade populacional (HALLORAN *et al*., 2016).

A alimentação das larvas é baseada em cereais ou grãos moídos. Assim, esse inseto é considerado uma praga de grãos e farelos armazenados (PHILIPS; THRONE, 2010). A presença de *T. molitor* pode contaminar os alimentos armazenados com fragmentos do corpo, fezes e indiretamente por microrganismos saprófitos, causando perda da qualidade desses alimentos (PLATA-RUEDA *et al*., 2017). As larvas também são usadas como alimento para vários animais domésticos ou zoológicos, incluindo peixes, lagartos e pássaros. Em muitos países, também são utilizadas para consumo humano. As larvas fornecem altos valores de proteínas e aminoácidos, que as tornam uma excelente fonte alternativa de alimentos. Além disso, apresentam uma taxa de crescimento relativamente alta e requerem pouca mão de obra e materiais leves para criação, o que reduz o tempo e os custos de trabalho (KIM *et al*., 2016; HICKS, 2017).

Os mecanismos do sistema humoral de *T. molitor* tem sido alvo de pesquisas, especialmente na síntese de peptídeos antimicrobianos, por possuírem características

semelhantes aos dos vertebrados. Este organismo exibe respostas imunes complexas com evidências de memória, demonstrando seu potencial como um modelo alternativo de infecção (MAISTROU *et al*., 2018).

Quando comparado a outros modelos alternativos de hospedeiros usados no estudo de patógenos humanos, o *T. molitor* possui algumas vantagens importantes. A larva pode ser incubada a 37 °C, o que, para muitos patógenos, é uma sugestão ambiental crítica para a expressão de fatores de virulência. Outros modelos, no entanto, não suportam temperaturas tão altas. *Drosophila melanogaster* sobrevive a uma temperatura de 18 a 30 °C, e a melhor temperatura para realizar testes com o verme *Caenorhabditis elegans* é de 15 a 25 °C. Uma segunda característica importante é o tamanho da larva. Como as larvas de *T. molitor* são relativamente grandes, isso permite a extração de um volume considerável de hemolinfa (5-10 µL) para análise. Isso representa uma vantagem sobre outros organismos modelo, como *D. melanogaster*, já que seu tamanho permite a extração de pequenos volumes de hemolinfa (0,05-0,3 µL). Outra vantagem é que o inóculo é administrado por injeção, permitindo a introdução direta na hemolinfa do animal. Além disso, as larvas mortas são facilmente identificadas porque ficam marrons através da melanização (CANTERI DE SOUZA *et al*., 2018).

3 RESULTADOS

3.1 ARTIGO 1

EVALUATION OF HEMOLYTIC, ANTITUMOR ACTIVITIES AND *Tenebrio molitor* TOXICITY OF ESSENTIAL OIL FROM *Eugenia gracillima* KIAERSK.

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Evaluation of hemolytic, antitumor activities and *Tenebrio molitor* toxicity of essential oil from *Eugenia gracillima* Kiaersk.

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Abstract

Essential oils (EOs) from plants used in the traditional medicine are known as a rich source of chemically diverse compounds with relevant biological properties. The aim of this study was to evaluate hemolytic and cytotoxic activities, and to determine the *Tenebrio molitor* toxicity of the *Eugenia gracillima* Kiaersk. essential oil (EgEO). EgEO was obtained from fresh leaves by the hydrodistillation method and chemical composition analyzed by GC/MS. Hemolytic activity was evaluated by spectrophotometric quantification of hemoglobin released by erythrocytes. Cytotoxic activity was determined by the MTT method. Contact toxicity was performed by the topical application of the essential oil to the larvae, pupae and adults of *T. molitor*. Lethal doses (LD_{50} and LD_{90}) were determined. The effects of EgEO on the development of *T. molitor* were evaluated after the injection of oil into the larvae (Sublethal doses: 4.6 and 2.3 mg/mL). A total of 39 components comprising 97.02% of EgEO was identified. All compounds identified belong to the sesquiterpene class. The essential oil under study showed low toxicity to human erythrocytes (Hemolysis: 0.29 - 10.47%). EgEO showed strong cytotoxicity for HL-60, MCF-7 and HT-29 cell lines, with IC_{50} values of 10.8, 14.0 and 35.0 μg/mL, respectively. The values of LD_{50} and LD_{90} indicated that pupae (LD_{50} = 4.55, LD_{90} = 13.60 mg/mL) were more susceptible to contact with EgEO followed by adults (LD_{50} = 5.24, LD_{90} = 13.40 mg/mL) and larvae (LD_{50} = 5.98, LD_{90} = 17.10 mg/mL). Sublethal doses of essential oil had no effect on the development of *T. molitor* and not induced deformities. This study reports, for the first time, the phytochemical characterization of the EgEO, as well as its *in vitro* and *in vivo* toxicity.

Keywords: Myrtaceae. Essential oils. Sesquiterpenes. Cytotoxicity. Mealworm.

Introduction

Plants have been used in the treatment of diseases since antiquity. Chemical compounds synthesized by plants such as alkaloids, glycosides, polyphenols and terpenoids have important biological properties (Salehi et al., 2018). Antimicrobial, antioxidant, anti-inflammatory and anti-tumor activities are some of the properties attributed to these phytochemicals (Sharopov et al., 2015; Ortega, Campos, 2019). However, the use of plants as therapeutic agents can cause adverse effects and even death (Ekor, 2014). Since the occurrence of adverse effects of phytomedication is common, due to inappropriate prescription, interaction with other bioactive compounds or overdose, besides the possibility of adulteration or contamination (Zhang et al., 2012). Many plants popularly used as therapeutic agents remain unassessed by scientific research to determine the pharmacological and toxicological aspects of its administration (Ahn, 2017).

Tenebrio molitor, also known as yellow mealworm, has been used as a model organism in diverse scientific researches. These animals have been increasingly chosen for research due to the absence of ethical restriction, ease of maintenance and short lifecycle. In addition, they have a complex innate immune system similar to vertebrates, consisting of humoral and cellular responses (Canteri de Souza et al., 2018).

Essential oils have been used in folk medicine throughout history. Studies confirm the biological properties of essential oils and propose different applications for them (Teixeira et al., 2013; Baser, Buchbauer, 2015). Thus, the toxicological characterization of essential oils is necessary to provide scientific data on the therapeutic safety of these products.

Eugenia gracillima Kiaersk., is a species of Myrtaceae, native and endemic to Brazil. It is present in different phytogeographical domains and the occurrence is confirmed in all regions of the country, except in the North (Sobral et al., 2015). Popularly known as “murta” in the Northeast of Brazil and as “cambuí” in the state of Paraná, this species produces fruits that can be consumed fresh or processed. In addition, the fruits present economic value for the region of the Chapada do Araripe, state of Pernambuco, Brazil (Araújo et al., 2016).

Toxicity tests are used to assess the toxic effects of substances in biological systems. The toxicity of the substances can be observed by *in vitro* studies using cells/cell lines and also by *in vivo* exposure in experimental animals. These *in vitro* and *in vivo* assays are considered

part of the preclinical developmental field and are generally performed to determine safe doses of exposure in humans (Parasuraman, 2011; Atanasov et al., 2015).

The aim of this study was to determine the chemical composition of essential oil obtained from the leaves of *E. gracillima* and to evaluate the cytotoxic and hemolytic activities by *in vitro* assays, as well as to investigate the toxicity *in vivo* in different stages of development of *T. molitor*.

Materials and Methods

Plant Material

Eugenia gracillima Kiaersk., was collected in the Chapada do Araripe, Pernambuco, Brazil (Latitude- 7° 49' 41" S; Longitude- 39° 51' 18" W), in July 2015. All the material used was processed following the usual techniques in taxonomy, being deposited in the IPA Herbarium (voucher access number: IPA 91.440), from the Agronomic Institute of Pernambuco.

Essential Oil Extraction

Essential oil extraction was performed at the Laboratory of Natural Products, Federal University of Pernambuco, Brazil. Fresh leaves of *E. gracillima* (370 g) were submitted to the hydrodistillation method, using a modified Clevenger apparatus, for a 4 h period (Guimarães et al., 2008). The essential oil of *E. gracillima* (EgEO) was then separated from the hydrolate by centrifugation at 3000 rpm for 5 min, using a benchtop centrifuge. EgEO was collected, packed in amber glass bottle and stored under refrigeration (4 °C). The EgEO yield was 1.32%.

Chemical Analysis of the Essential Oil

Gas chromatography

Quantitative GC analyses were carried out using a Shimadzu GC-QP2010 Ultra apparatus equipped with a flame ionization detector (FID) and a non-polar column Rxi®-5ms (Crossbond® 5% diphenyl / 95% dimethylpolysiloxane) (10m x 0,10 mm ID x 10µm df). The oven temperature was programmed from 40 to 250°C at a rate 25°C/min for integration purposes. Injector and detector temperatures were 260°C. Hydrogen was used as the carrier gas at a flow rate of 1 L/min and 30 p.s.i. inlet pressure in split mode (1:30). The injection volume was 0.5 µL of diluted solution (1/100) of oil in n-hexane. The amount of each compound

was calculated from GC peak areas in the order of DB-5 column elution and expressed as a relative percentage of the total area of the chromatograms. Analyses were carried out in triplicate.

Gas chromatography-mass spectrometry

The qualitative GC/MS analysis were carried out using a analyses were performed using a Shimadzu GCMS-QP2010 Ultra system with a mass selective detector, mass spectrometer in EI 70 eV with a scan interval of 0.5 s and fragments from 40 to 550 Da. fitted with the same column and temperature program as that for the GC experiments, with the following parameters: carrier gas = helium; flow rate = 1 mL/min; split mode (1:30); injected volume = 1 µL of diluted solution (1/100) of oil in n-hexane.

Identification of componentes

Identification of the components was based on GC retention indices with reference to a homologous series of C8-C40 n-alkanes calculated using the Van Den Dool and Kratz equation (Van Den Dool and Kratz, 1963) and by computer matching against the mass spectral library of the GC/MS data system (NIST 98 and WILEY) and co-injection with authentic standards as well as other published mass spectra (Adams, 2007). Area percentages were obtained electronically from the GC-FID response without the use of an internal standard or correction factors.

In vitro Analysis

Hemolytic assay

The evaluation of hemolytic activity of EgEO on human red blood cells was performed as described by Ahmad et al. (2010), with modifications. The blood (O^+) used in experiment was collected in Vacutainer® tubes containing sodium heparin.

The fresh blood (10 mL) was centrifuged (206 BL FANEM Baby®I centrifuge) for 10 minutes at 1200 rpm. After plasma removal, the pellet containing the erythrocytes was washed three times with PBS (pH 7.4, 5mM) and then resuspended in PBS to obtain an 8% (v/v) suspension. Then 100 µL of this suspension was added to different microcentrifuge tubes with 100 µL of twofold serial dilutions of essential oil (0.11–1.76 mg/mL). Final concentration of erythrocyte suspension was 4% (v/v). The resulting suspensions were incubated with agitation for 60 minutes at 37 ºC. After incubation, the samples were centrifuged for 5 minutes at 1500

rpm. The supernatants (200 µL) were transferred to 96-well plates and the concentration of hemoglobin was determined spectrophotometrically (Shimadzu UV-160 1 PC) at 540 nm. The negative and positive controls were the erythrocytes in PBS and the erythrocytes in Triton X-100 at 1%, respectively. Measurements were performed in triplicate for each oil concentration and for the following controls: (c1) erythrocytes in PBS (negative control) and (c2) erythrocytes in Triton X-100 at 1% (positive control). Percentage hemolysis was determined according to the equation below:

$$\% \text{ Hemolysis} = [(Aa - Ac1)/(Ac2 - Ac1)] \times 100$$

where Aa, Ac1 and Ac2 are, respectively, the absorbance of the sample and controls c1 and c2 at 540 nm.

Antitumor assay

The evaluation of cytotoxic activity was performed by MTT colorimetric method [3-(4, 5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide] (Mosmann, 1983; Alley et al., 1988). The following cells were used for the cytotoxicity assay: NCI-H292 (human lung mucoepidermoid carcinoma), MCF-7 (human breast adenocarcinoma), HL-60 (acute promyelocytic leukemia) and HT-29 (human colon adenocarcinoma). All cell lines were obtained from the Cell Culture Laboratory of the Department of Antibiotics of UFPE, Brazil. The NCI-H292 and HT-29 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (SIGMA). The MCF-7 and HL-60 tumor cells were kept in the RPMI 1640 culture medium created by the Roswell Park Memorial Institute. The media were supplemented with 10% fetal bovine serum (GIBCO) and a 1% antibiotic solution (penicillin and streptomycin). The cells were kept at 37 ºC in a humid atmosphere enriched with 5% CO_2.

The NCI-H292, MCF-7, HT-29 (10^5 cells/mL) and HL-60 (3×10^5 cells/mL) cells were plated (190 µL) in 96-well plates and incubated for 24 h. Next, 10 µL of the essential oil dissolved in DMSO (0.5%) were added to the wells at final concentration of 50 µg/mL. The drug doxorubicin at a concentration of 1.25 µg/mL was used as the positive control and DMSO (0.5%) was used as the negative control. After 72 h of incubation, 25 µL of MTT (5 mg/mL) were added and the plates were incubated again for 3 h, after which the culture medium with the MTT was aspirated and 100 µL of DMSO were added to each well. Absorbance was read in a microplate reader at a wavelength of 560 nm. The experiments were conducted in quadruplicate.

An intensity scale was used to evaluate the cytotoxic potential of the samples based on the percentage of growth inhibition: 95-100% = high potential; 70-90% = moderate potential; < 50% = no potential (Rodrigues et al, 2014). The concentration that inhibits growth by 50% in comparison to the negative control (IC_{50}) was determined considering moderate inhibitory activity (70%) or higher.

In vivo Analysis

Tenebrio molitor

T. molitor larvae were purchased from a commercial supplier (Insetos Brasil – Recife, Pernambuco, Brazil) and kept in plastic trays (60 cm long×40cm wide×12cm) at 26 ºC ± 1 in a 12:12 h light/dark cycle. These individuals were fed *ad libitum* with wheat bran (12% protein, 2% lipids, 75% carbohydrates and 11% mineral/sugar), pieces of sugarcane and chayote. Healthy larvae (last instar larval), pupae, and adults of *T. molitor* of 48 h old were chosen for the bioassays.

Contact toxicity

The assay was performed according to Plata-Rueda et al. (2017), with modifications. A serial dilution of the EgEO (concentrations of 18.4, 9.2, 4.6, 2.3 and 1.1 mg/mL) was prepared in sterile saline solution with DMSO (0.5%) and tween 20 (1%). Aliquots of 0.5 µL of the dilutions were applied topically to the abdominal segments of larva, pupa and adult of *T. molitor*, using a micropipette. Sterile saline solution with DMSO (0.5%) and tween (1%) was used as negative control. The individuals were placed in Petri dishes (Ø 90 mm×15 mm) (10 individuals/Petri dish) with an absorbent paper, fed with wheat bran and chayote *ad libitum* (larvae and adults) and maintained in the dark. A total of 30 larvae, 30 pupae, and 30 adults were used for each treatment and the number of dead individuals was counted after 48 h. The experiment was performed in triplicate. Lethal doses required to kill 50% and 90% of the individuals (LD_{50} and LD_{90}, respectively) were determined for each stage of development of *T. molitor*.

Effect of the EgEO on the development of T. molitor

T. molitor larvae were iced for 2 min to stop their movement and 1 µL of the EgEO (concentrations of 4.6 and 2.3 mg/mL) was injected between the 4th and 5th abdominal sternites, using a 1 mL syringe. Sterile saline solution with DMSO (0.5%) and tween (1%) was

used as negative control. After application, larvae were placed in Petri dishes (Ø 90 mm×15 mm) (10 individuals/Petri dish) with an absorbent paper, fed with wheat bran and chayote *ad libitum* and maintained in the dark. For each treatment, 30 larvae were used. The number of larvae that successfully pupated, as well as the duration of the pupal stage (in days) were recorded every 24 h for 30 days. The newly emerged adults of *T. molitor* were evaluated for possible morphological abnormalities (deformations in elytra, wings, antennae, legs and abdominal segments) (Steinhaus, Zeikus, 1968). The experiment was performed in triplicate.

Statistical Analysis

Data were expressed as mean ± standard deviation. Statistical analysis and graphical representation of the data were performed using *GraphPad Prism* 7.0 (GraphPad Software). Data were analyzed using ANOVA. The IC_{50}, LD_{50} and LD_{90} values and respective 95% confidence intervals (CI) were determined by nonlinear regression. P values < 0.05 were considered statistically significant.

Results

A total of 39 components comprising 97.02% of the EgEO was identified (Table 1). All compounds identified belong to the sesquiterpene class. Allo-aromadendrene (16.37%), valencene (14.59%), *α*-chamigrene (7.05%), *γ*-himachalene (6.11%), *δ*-elemene (5.89%) and viridiflorene (5.60%) were identified as the major constituents of the EgEO.

The assay to determine the *in vitro* hemolytic activity of EgEO showed that at concentracions of 0.11, 0.22, 0.44, 0.88 and 1.76 mg/mL, the hemolysis percentage was 0.29, 0.87, 2.25, 4.78 and 10.47%, respectively (Figure 1).

Table 2 displays the inhibitory capacity of the EgEO and doxorubicin (positive control) regarding the growth of the tumor cells. The EgEO demonstrated high inhibitory capacity (cell growth inhibition ranging from 95 to 100%) toward the HL-60 cell line, moderate inhibitory capacity (cell growth inhibition ranging from 70 to 90%) toward the MCF-7 and HT-29 cell lines, but exhibited no cytotoxic potential with regard to NCI-H292.

EgEO that exhibited moderate to high inhibitory activity were tested at different concentrations (0.098 to 50 µg/ mL) for the determination of IC_{50} values. Doxorubicin (0.009 to 1.25 µg/mL) was used as the standard.

Table 3 displays the IC_{50} values of the EgEO. The EgEO exhibited cytotoxic potential against the neoplastic cell lines tested (IC_{50} < 50 µg/mL). IC_{50} values obtain to HL-60, MCF-7 and HT-29 cell lines were 10.8, 14.0 and 35.0 µg/mL, respectively. Therefore, EgEO was more effective at exerting cytotoxic effects on the HL-60 cell line (IC_{50} = 10.8 µg/mL).

The toxicity of EgEO was evalute also *in vivo*, using *T. molitor* as organism model. The contact toxicity of EgEO was performed for different stage of development of the insect. Table 4 displays the LD_{50} and LD_{90} values of EgEO. The LD_{50} and LD_{90} values indicated that the pupae (LD_{50} = 4.55; LD_{90} = 13.60 mg/mL) were more susceptible to contact with EgEO followed by adults (LD_{50} = 5.24; LD_{90} = 13.40 mg/mL) and larvae (LD_{50} = 5.98; LD_{90} = 17.10 mg/mL).

Table 5 displays the effect of the EgEO on the development of *T. molitor*. EgEO did not cause changes in the number of larvae that reached pulpation. In addition, the essential oil did not affect the viability and emergence of the pupae.

The newly emerged adults of *T. molitor* did not present morphological abnormalities, such as deformations in elytra, wings, antennae, legs and abdominal segments.

Discussion

Sesquiterpenes are commonly found in essential oils obtained from *Eugenia* genus. Some sesquiterpenes, such as allo-aromadendrene and β-elemene, identified in EgEO are characteristic of the genus (Tenfen et al., 2015). In previous studies with essential oils of others *Eugenia* species, like *E. luschnathiana* (Monteiro et al., 2016), *E. brejoensis* (Oliveira de Souza et al., 2017), *E. egensis* and *E. flavescens* (Da Silva et al., 2017), only sesquiterpenes were identified in the chemical analysis.

Sampaio et al. (2019) identified sesquiterpene hydrocarbons (91.22%), oxygenated sesquiterpenes (7.45%) and monoterpene (1.01%) in EgEO. The most abundant volatile constituents of the EgEO were germacrene D (16.10%), c-muurolene (15.60%), bicyclogermacrene (8.53%), germacrene B (7.43%), and D-elemene (6.06%).

Sesquiterpenes have many biological properties and, particularly, cytotoxic activity has been reported with promising results against tumor cells (Sharifi-Rad et al., 2017). According to a review of the literature by Bosco and Golsteyn (2017), the most common biological activity investigated with sesquiterpenes was that of cytotoxicity. In addition, several compounds of the

sesquiterpene class have demonstrated selective cytotoxicity against cancer cells, which is quite relevant in the search for new antitumor agentes (Yeo et al., 2015).

Methods for evaluate the hemolytic activity consists of checking for potential damage caused by a natural product or drug to the membranes of erythrocytes (Miyazaki et al., 2014). The hemolytic action of a drug can occur by various mechanisms, from the dissolution or increase of the permeability of the cellular membranes until the complete cellular lysis (Mortazavian et al., 2018).

The high lipid composition of erythrocyte membranes allows the interaction of lipophilic compounds, such as those present in essential oils with the erythrocyte membrane, increasing its permeability and resulting in cell lysis. Therefore, this has been the mechanism most commonly associated with hemolysis caused by essential oils (Barros et al., 2016).

Considering the results obtained in this research, it is possible to infer that the studied essential oil presents low toxicity to erythrocytes at the concentrations tested, since percentages of hemolysis above 15% are considered high, corresponding to extensive damage to erythrocyte membranes (Preté et al., 2011).

Based on US National Cancer Institute standards, an extract has high potential to be developed as an anticancer agent if it has a 50% inhibitory concentration (IC_{50}) < 50 μg/mL (Susianti et al., 2018). Thus, according to the IC_{50} values obtained in this study, EgEO has a strong cytotoxic effect on the tumor cells investigated and potential to be developed as an anticancer substance.

Recent studies have also reported the cytotoxicity properties of essential oils of *Eugenia* species with IC_{50} values similar to those found in this research. Da Silva et al. (2017) reported the cytotoxic potential of three *Eugenia* essential oils to HCT-116 cells (colon cancer) by the MTT assay, where the most active was *E. polystachya* oil (IC_{50} = 10.3 μg/mL), followed by *E. flavescens* (IC_{50} = 13.9 μg/mL) and *E. patrisii* (IC_{50} = 16.4 μg/mL). In addition, the authors identified that sesquiterpenes were the class of compounds most represented in these oils (*E. polystachya*, *E. flavescens* and *E. patrisii* presented 94.3, 96.3 and 95.2% of sesquiterpernes, respectively).

Figueiredo et al. (2019) reported that essential oils of two chemotypes of *E. uniflora* with high content of sesquiterpenes (90.4 and 84.4%) have cytotoxic activity against HCT-116 (colon) (IC_{50}=16.26 μg/mL; IC_{50}= 9.28 μg/mL), AGP-01 (malignant gastric ascites)

(IC_{50}=12.60 μg/mL; IC_{50}=8.73 μg/mL) and SKMEL-19 (melanoma) (IC_{50}=12.20 μg/mL; IC_{50}=15.42 μg/mL) cancer cell lines.

Martínez et al. (2017) evaluated the contact toxicity of cinnamon and clove essential oils, also in the *T. molitor*. As in our study, the pupae (LC_{50} = 10.7 and 6.45 μg insect^{-1}, respectively) were more susceptible to contact with the essential oils followed by adults (LC_{50} = 29.8 and 21.6 μg insect^{-1}, respectively) and larvae (LC_{50} = 30.4 and 35.1 μg insect^{-1}, respectively). However, Plata-Rueda et al. (2017) reported that larvae (LC_{50} = 0.77; LC_{90} = 1.36%) were more susceptible to contact with the garlic essential oil, followed by pupae (LC_{50} = 2.37; LC_{90} = 4.01%) and adults (LC_{50} = 2.03; LC_{90} = 4.73%).

One possible explanation for the developmental stages difference is that potency may be affected by the penetration of the essential oils compounds into the body and the ability of the insect to metabolize these compounds (Plata-Rueda et al., 2017).

In contrast to our results, the studies mentioned indicated that small amounts of the essential oils tested were toxic in *T. molitor.*

EgEO did not cause changes in the number of larvae that reached pulpation and did not affect the viability and emergence of the pupae. The newly emerged adults of *T. molitor* did not present morphological abnormalities. Therefore, EgEO at sublethal concentrations did not affect the life cycle or induce deformities in *T. molitor*.

Conclusions

The essential oil from *E. gracillima* leaves is rich in sesquiterpenes. In physiological conditions the oil showed low cytotoxic to human erytrocitys, and concentration-dependent effects were observed. The cytotoxic activity of EgEO on cancer cells was considered high, particulary against HL-60 line. LD values obtained to showed that the pupae of *T. molitor* were more susceptible to toxicity contact with EgEO. In sublethal concentrations the oil did not caused deformities in *T. molitor.* Data obtained in this research suggest the antitumoral potential of the EgEO and low toxic potential.

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Tables

Table 1. Chemical compounds identified in the *Eugenia gracillima* essential oil (EgEO) by GC-MS.

	Compounds	IR[a]	IR[b]	%
1	*δ*-elemene	1335	1330	5.89
2	*α*-cubebene	1348	1346	0.22
3	*α*-copaene	1374	1374	0.84
4	*β*-cubebene	1387	1385	0.73
5	*β*-bourbonene	1387	1387	0.99
6	*β*-elemene	1389	1390	1.89
7	*β*-caryophyllene	1417	1413	3.64
8	*β*-ylangene	1419	1418	0.87
9	*β*-duprezianene	1421	1420	1.18
10	4,8-*β*-epoxy-caryophyllene	1423	1422	0.33
11	(*E*)-*α*-Ionene	1428	1416	1.18
12	*β*-copaene	1430	1429	1.07
13	*β*-gurjunene	1431	1430	0.28
14	Aromadendrene	1439	1436	1.46
15	α-neo-clovene	1452	1453	2.15
16	Allo-aromadendrene	1458	1455	16.37
17	9-epi-(E)-caryophyllene	1464	1464	1.39
18	*γ*-gurjunene	1475	1473	0.96
19	*γ*-himachalene	1481	1482	6.11
20	*α*-amorphene	1483	1483	0.90
21	Germacrene D	1484	1485	0.23
22	*δ*-Selinene	1492	1490	3.72
23	Valencene	1496	1495	14.59
24	Viridiflorene	1496	1496	5.60
25	α-Muurolene	1500	1499	0.62
26	Epozonarene	1501	1501	0.27
27	*Trans*-*β*-guaiene	1502	1502	2.45
28	*α*-chamigrene	1503	1503	7.05
29	*β*-bisabolene	1505	1506	0.77
30	Germacrene A	1508	1509	0.56
31	α-bulnesene	1509	1510	0.76
32	*δ*-Amorphene	1511	1511	1.40
33	*γ*-Cadinene	1513	1513	4.05
34	(*Z*)-*γ*-bisabolene	1514	1514	0.95
35	Cubebol	1514	1515	2.23
36	Nootkatene	1517	1518	0.30
37	7-epi-α-selinene	1520	1521	0.61
38	Trans-calamenene	1522	1523	0.52
39	*δ*-Cadinene	1522	1525	1.39
	Sesquiterpenes			97.02
	Total			97.02

[a]Linear retention indices from the literature. [b]Retention indices calculated from retention times in relation to those of a series of n-alkanes on a 30 m DB-5 capillary column.

Table 2. Percentage of inhibition to growth of human tumor cell.

Samples	Concentration (µg/mL)	% of inhibition			
		NCI-H292	MCF-7	HL-60	HT-29
EgEO	50	23.4 ± 0.3	91.2 ± 5.2	97.6 ± 2.2	90.4 ± 7.1
Doxorubicin	5	94.1 ± 1.9	79.4 ± 2.6	92.9 ± 0.6	64.1 ± 1.1

EgEO = *Eugenia gracillima* essential oil. Data expressed as mean and standard deviation. The tests were done in quadruplicate and in 3 different experiments.

Table 3. IC_{50} of the *Eugenia gracillima* essential oil (EgEO) against the tumor cell lines.

Samples	Concentrations (µg/mL)	IC_{50} (µg/mL)			
		NCI-H292	MCF-7	HL-60	HT-29
EgEO	0.098-50	NT	14.0 12.0-16.4	10.8 9.8-11.8	35.0 30.1-40.7
Doxorubicin	0.009-1.25	0.08 0.06-0.12	0.11 0.08-0.15	0.06 0.05-0.07	1.83 1.36-2.40

EgEO = *Eugenia gracillima* essential oil; NT = not tested. The tests were done in quadruplicate and in 3 different experiments. The values were expressed as IC_{50} (concentration that inhibited cell proliferation by 50%) and confidence interval using GraphPad Prism 7.0 Demo.

Table 4. Lethal doses of the *Eugenia gracillima* essential oil (EgEO) on different developmental stages of *Tenebrio molitor* after 48 hours exposure.

Insect stage	[1]LD	[2]EV	[3]IC (mg/mL)
Larva	LD_{50}	5.98	5.72 – 6.23
	LD_{90}	17.10	16.83 – 17.36
Pupa	LD_{50}	4.55	4.41 – 4.68
	LD_{90}	13.60	13.09 – 14.10
Adult	LD_{50}	5.24	5.03 – 5.44
	LD_{90}	13.40	12.94 – 13.85

[1]$LD_{50 \text{ and } 90}$, lethal dose causing 50 and 90% mortality; [2]EV, estimated value; [3]CI, confidential interval.

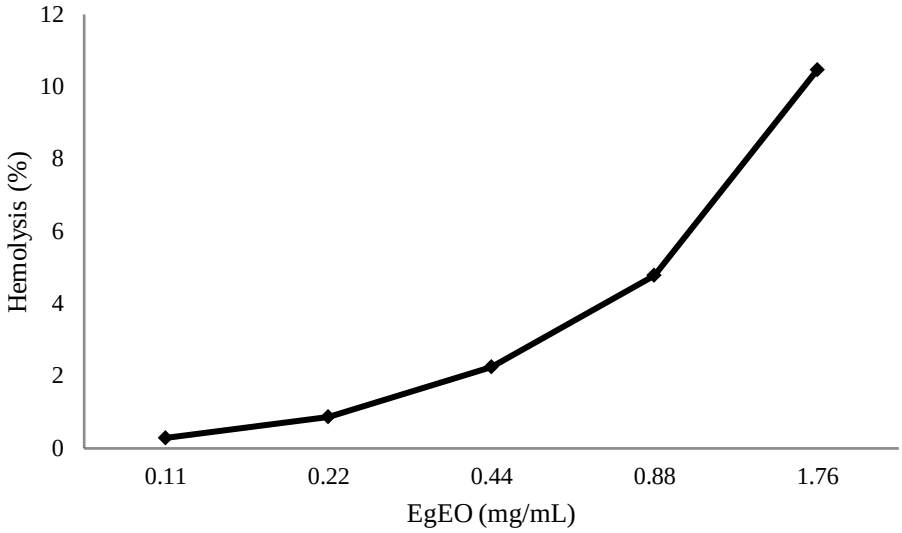
Table 5. Effect of the *Eugenia gracillima* essential oil (EgEO) on the development of *Tenebrio molitor*.

Treatments	Number of pupae formed				[a]Duration of pupal stage	[b]Successfully pupation %	[c]Emergence %
	5 Days	10 Days	20 Days	25 Days			
T1	7	13	45	82	14.5 ± 0.8	91	91
T2	5	12	47	85	15.5 ± 0.2	94	93
Control	6	12	46	87	15.5 ± 0.6	97	97

[a]Average duration, the criteria used were to measure until emergence of survival pupae. Data expressed as mean and standard deviation. [b]Percentage with respect to control. [c] % = Number of adults emerged x 100/Total number of pupae.

Figures

Figure 1. Percentage of hemolysis induced by different concentrations of the *Eugenia gracillima* essential oil (EgEO).



3.2 ARTIGO 2

ANTI-*Staphylococcus aureus* ACTIVITY OF *Eugenia gracillima* KIAERSK. ESSENTIAL OIL: ANTI-VIRULENCE ACTION AND *in vivo* EVALUATION IN *Tenebrio molitor* INFECTION MODEL.

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Anti-*Staphylococcus aureus* activity of *Eugenia gracillima* Kiaersk. essential oil: anti-virulence action and *in vivo* evaluation in *Tenebrio molitor* infection model

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Abstract

This work evaluated the activity of the essential oil of *Eugenia gracillima* Kiaersk. (EgO) against standard and multiresistant strains of *Staphylococcus aureus in vitro* and *in vivo* using the *Tenebrio molitor* model. The chemical characterization of EgEO was performed for the first time using the GC / MS technique. It was observed that all the compounds identified (97.02%) belong to the class of sesquiterpenes. The antimicrobial potential of EgEO was determined by the broth dilution method, in which MIC and MBC values were obtained. EgEO inhibited the growth of all strains tested (MIC 0.01 - 1.84 mg/mL) and showed bactericidal activity for seven of the strains tested (MBC 0.46 - 1.84 mg/mL). In the time-kill assay, concentrations of EgEO corresponding to 2xMIC for strains ATCC 29213 (0.92 mg/mL) and UFPEDA 683 (0.02 mg/mL) inhibited the growth of *S. aureus* in less time when compared to concentrations corresponding to 1/2 MIC and MIC in all strains, demonstrating a dose-dependent relationship of EgEO over time. The staphyloxanthin (STX) biosynthesis was determined by absorbance at 465 nm, and EgEO demonstrated anti-virulence potential, reducing the production of this carotenoid at sub-MIC and MIC concentrations. At a concentration previously determined as sublethal (4.612 mg/mL), the essential oil was effective against *S. aureus* infection in *T. molitor* only in the standard strain. This is the first report of the application of this oil in the antimicrobial activity associated with the anti-virulence action, as well as using a model of infection of *T. molitor*.

Keywords: Myrtaceae. Sesquiterpenes. Time-kill assay. Staphyloxanthin.

Introduction

Resistance to antibiotics has become a public health problem on a global scale. In 2014, the World Report on Antimicrobial Resistance of the World Health Organization (WHO) considered that the growth of antibiotic resistance is a threat to global health (WHO, 2014). Considering that microbial resistance represent a challenge in the treatment of infections, it is evident the need to find new substances to be used in the control of these microorganisms.

Staphylococcus aureus is a commensal bacterium and an important etiological agent associated with nosocomial and community-acquired infections (Tong et al., 2015). It is characterized by its high frequency and pathogenicity (Aryee and Edgeworth, 2016). In addition, this microorganism is associated with multiple antibiotic resistance. The first strains of methicillin-resistant *S. aureus* (MRSA) emerged in the 1960s, and have since become a challenge for conventional antimicrobial treatment (Stryjewski and Corey, 2014).

Recently, the World Health Organization (WHO) has published a list of priority pathogens according to the need for the development of new antibiotics. In this list, antibiotic-resistant microorganisms, such as *Klebsiella pneumoniae* and *Escherichia coli*, were classified at critical, high and medium levels, and MRSA is described in the group considered high (WHO, 2017).

Medicinal and aromatic plants are considered an important resource for the pharmaceutical, food, cosmetics and perfumery industries. The discovery of new medicines is related to the research of natural products, considering that a large part of the world population depends on plant-based medicines for the treatment of several pathologies (Swamy and Sinniah, 2015). Essential oils obtained from medicinal and aromatic plants are complex volatile compounds consisting of a mixture of substances including terpenes, aldehydes, esters, ethers, phenols, alcohols and ketones. Biological activities such as antimicrobial, antioxidant, antitumor and anti-inflammatory are attributed to essential oils (Zhang et al., 2017; Nascimento et al., 2018).

The antimicrobial properties of essential oils depend mainly on their chemical constitution. The presence of different types of terpenes, phenols, aldehydes and other antimicrobial compounds justifies the diversified action of essential oils against bacterial, fungal and viral pathogens (Akhtar et al., 2014). Essential oils have demonstrated high potential

against pathogenic microorganisms of clinical interest like *Escherichia coli, S. aureus, Candida albicans, Candida glabrata* and Influenza A virus (H5N1 e H2N2) (Swamy et al., 2016).

Eugenia gracillima Kiaersk. (Myrtaceae) is a native and endemic species of Brazil. The plant is a shrub that varies in height from 1.5 to 5.0 meters. Known popularly as "murta" in Northeastern Brazil or as "cambuí" in other regions, its fruits are consumed fresh or processed in the form of pulp, juice, jelly, liquor or desserts. In the Brazilian northeast, the fruits of the species have socioeconomic importance (Araujo et al., 2016).

The aim of this work was to evaluate the activity of essential oil obtained from leaves of *Eugenia gracillima* Kiaersk. against standard and multiresistant strains of *Staphylococcus aureus*, investigating the anti-virulence potential and *in vivo* antimicrobial activity.

Materials and Methods

Plant Material

Eugenia gracillima Kiaersk., was collected in the Chapada do Araripe, Pernambuco, Brazil (Latitude- 7° 49' 41" S; Longitude- 39° 51' 18" W), in July 2015. All the material used was processed following the usual techniques in taxonomy, being deposited in the IPA Herbarium (voucher access number: IPA 91.440), from the Agronomic Institute of Pernambuco.

Essential Oil Extraction

Essential oil extraction was performed at the Laboratory of Natural Products, Federal University of Pernambuco, Brazil. Fresh leaves of *E. gracillima* (370 g) were submitted to the hydrodistillation method, using a modified Clevenger apparatus, for a 4 h period (Guimarães et al., 2008). The essential oil of *E. gracillima* (EgEO) was then separated from the hydrolate by centrifugation at 3000 rpm for 5 min, using a benchtop centrifuge. EgEO was collected, packed in amber glass bottle and stored under refrigeration (4 °C). The EgEO yield was 1.32%.

Chemical Analysis of the Essential Oil

Gas chromatography

Quantitative GC analyses were carried out using a Shimadzu GC-QP2010 Ultra apparatus equipped with a flame ionization detector (FID) and a non-polar column Rxi®-5ms (Crossbond® 5% diphenyl / 95% dimethylpolysiloxane) (10m x 0,10 mm ID x

10µm df). The oven temperature was programmed from 40 to 250°C at a rate 25°C/min for integration purposes. Injector and detector temperatures were 260°C. Hydrogen was used as the carrier gas at a flow rate of 1 L/min and 30 p.s.i. inlet pressure in split mode (1:30). The injection volume was 0.5 µL of diluted solution (1/100) of oil in n-hexane. The amount of each compound was calculated from GC peak areas in the order of DB-5 column elution and expressed as a relative percentage of the total area of the chromatograms. Analyses were carried out in triplicate.

Gas chromatography-mass spectrometry

The qualitative GC/MS analysis were carried out using a analyses were performed using a Shimadzu GCMS-QP2010 Ultra system with a mass selective detector, mass spectrometer in EI 70 eV with a scan interval of 0.5 s and fragments from 40 to 550 Da. fitted with the same column and temperature program as that for the GC experiments, with the following parameters: carrier gas = helium; flow rate = 1 mL/min; split mode (1:30); injected volume = 1 µL of diluted solution (1/100) of oil in n-hexane.

Identification of componentes

Identification of the components was based on GC retention indices with reference to a homologous series of C8-C40 n-alkanes calculated using the Van Den Dool and Kratz equation (Van Den Dool and Kratz, 1963) and by computer matching against the mass spectral library of the GC/MS data system (NIST 98 and WILEY) and co-injection with authentic standards as well as other published mass spectra (Adams, 2007). Area percentages were obtained electronically from the GC-FID response without the use of an internal standard or correction factors.

Antimicrobial Activity

Bacterial strains

Strains of the *Staphylococcus aureus* ATCC 29213, UFPEDA 02 and clinical isolates: UFPEDA 659/670/671/679/683/691/705/726/731/802 were provided by the Department of Antibiotics, UFPE, Brazil.

Disc diffusion test - Resistance profile

To evaluate the resistance profile of the strains of *S. aureus,* disc diffusion test was performed according to CLSI (2014) using the standard antibiotics: ampicillin (AMP), oxacilin

(OXA), cephalotin (CFL), cephoxitin (CFO), cephazoline (CFZ), cefepime (CPM), cefuroxime (CRX), cefotaxime (CTX), imipenem (IPM), meropenem (MER), nalidixic acid (NAL), ciprofloxacin (CIP), nitrofurantoin (NIT), vancomycin (VAN), amikacin (AMI), gentamicin (GEN), clindamycin (CLI), chloramphenicol (CLO), tetracycline (TET), trimetropim (TRI).

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Minimum inhibitory concentration (MIC) was measured by the microdilution method (CLSI, 2015). A twofold serial dilution of the EgEO was prepared in Luria Bertani (LB) broth in 96 well microplates. Then, 10 µL of the bacterial suspension (OD_{600} 0,15 ± 0,05) was added. The plates were incubated for 24 h at 37°C and the growth rate were obtained through the OD_{600} after incubation. To measure the minimum bactericidal concentration (MBC), the content from the wells (5 µL) was cultured on Muller Hinton (MH) agar and incubated for 24 h at 37°C.

The minimum inhibitory concentration (MIC) was determined as the lowest concentration of EgEO in which there was a ≥ 50% reduction in optical density (bacterial growth) relative to OD_{600} in negative control. The MBC value was characterized as the lowest concentration that eliminated the bacteria. All experiments were performed in quadruplicate.

Time-kill assay

The time-kill assay was performed with the ATCC 29213 and UFPEDA 683 strains. Bacterial culture (100 µL; OD_{600} 0.15 ± 0.05 in saline) was incubated in LB broth (900 µL) containing different concentrations of EgEO (MIC/2, MIC and 2xMIC). Bacterial growth was evaluated considering the following intervals: 0, 1, 2, 3, 4, 5, 6 and 24 h and for couting viable cells in plate agar after incubation at 37°C for 24 h. Bacterial growth was expressed by reduction in CFU/mL. All experiment was performed in triplicate.

Staphyloxanthin assay

The staphyloxanthin assay was performed with the ATCC 29213, UFPEDA 02, UFPEDA 802 and UFPEDA 683 strains. The production of *S. aureus* staphyloxanthin (STX) was performed as described by Lee et al. (2013) slightly modified. *S. aureus* cells were reinoculated at 1:100 dilution in MH medium (20 mL) and aliquots (100 µL) of this suspension were incubated with diferent concentrations (sub-MIC and MIC) of EgEO at 37°C for 24 h in eppendorfs at 200 rpm, in quadruplicate. EgEO was not added in the control. Cells were

collected by centrifugation at 9000 rpm for 10 min and washed once with saline. STX was extracted with MeOH (600 µL) and the OD was determined at 465 nm. All experiment was performed in quadruplicate.

In vivo assays

Tenebrio molitor

T. molitor larvae were purchased from a commercial supplier (Insetos Brasil – Recife, Pernambuco, Brazil) and kept in plastic trays (60 cm long×40cm wide×12cm) at 26 ºC ± 1 in a 12:12 h light/dark cycle. These individuals were fed *ad libitum* with wheat bran (12% protein, 2% lipids, 75% carbohydrates and 11% mineral/sugar), pieces of sugarcane *Saccharum officinarum* (L.) (Poaceae) and chayote *Sechium edule* (Jacq.) Swartz (Cucurbitaceae) (Plata-Rueda et al., 2017). Healthy larvae (last instar larval) were chosen for the bioassays.

T. molitor x *S. aureus* infection

S. aureus was cultured in Luria-Bertani (LB) broth overnight at 37°C. The cells were centrifuged at 3500 rpm for 10 min. The bacterial pellet was washed twice in phosphate-buffered saline (PBS) and diluted with PBS to reach a concentration of 10^2 cells/mL. *T. molitor* larvae were microinjected with 1 µL suspensions of *S. aureus* (10^2 cells/mL) using a 1 mL syringe. After 2 h of infection, the larvae were treated with EgEO (concentrations previously determined as sublethal: 1.153, 2.302, 4.612 mg/mL). The control larvae were injected with 1 µL of PBS at the same time. Following this, larvae were maintained under standard rearing conditions in isolation. A total of 30 larvae per treatment (n = 10) were used and the results represent an average of three biological replications.

Statistical Analysis

Data were expressed as mean ± standard deviation. Statistical analysis and graphical representation of the data were performed using *GraphPad Prism* 7.0 (GraphPad Software). Data were analyzed using ANOVA. P values < 0.05 were considered statistically significant.

Results and Discussion

In the chemical analysis of EgEO, 39 compounds were identified, representing 97.02% of the total oil content (Table 1). All compounds identified in EgEO belong to the class of sesquiterpenes. The major compounds were allo-aromadendrene (16.37%), valencene

(14.59%), α-chamigrene (7.05%), γ-himachalene (6.11%), δ-elemene (5.89%) and viridiflorene (5.60%).

In the literature there are reports of other species of the family Myrtaceae in which the composition of the essential oils analyzed was rich in sesquiterpenes. Studies with essential oils of *E. punicifolia* and *E. luschnathiana* identified, respectively, 98.2% and 97.2% of the compounds and all belonged to the class of sesquiterpenes (Ramos et al., 2010; Monteiro et al., 2016). Godinho et al. (2014) observed the predominance of sesquiterpenes (96.53%) in the essential oil of *Blepharocalyx salicifolius*, considering the total percentage of compounds identified (97.82%). A recent study reported that the essential oil of *E. candolleana* is composed mainly of sesquiterpenes (94.21%), in addition the compounds δ-elemene (8.28%) and viridiflorene (6.96%) were among the major compounds, as in EgEO used in our study (Neves et al., 2017). The allo-aromadendrene, identified in this study as a major component of EgEO, has not been described among the major components of the essential oils of these species. The mentioned studies demonstrate a chemical profile similar to that presented in this study, showing the sesquiterpenes as predominant compounds of these essential oils.

Sesquiterpenes exhibit a variety of biological properties, such as antimicrobial, antioxidant, anticancer and anesthetic, and therefore have high pharmaceutical interest (Dahham et al., 2015; Fu et al., 2017). The antimicrobial potential of these compounds has been evaluated against several pathogens of clinical interest, such as *S. aureus*, *Escherichia coli* and *Candida albicans* (Santoso et al., 2018). In the literature, the anti-*S. aureus* activity of isolated sesquiterpenes has been reported (Liu et al., 2015; Jiang et al., 2017). In this study, we evaluated the anti-*S. aureus* potential of EgEO considering the composition rich in sesquiterpenes.

S. aureus is an important opportunistic pathogen that can cause infections ranging from skin disorders to systemic diseases. Due to the multiple antimicrobial resistance developed by this pathogen, the currently available therapeutic options are often inefficient (Anstead et al., 2014; Oliveira et al., 2018). In view of the need to develop new antimicrobials, bioactive compounds present in medicinal and aromatic plants have been widely investigated as potentially applicable substances for the treatment of infections (Merghni et al., 2018). Essential oils have shown promising results against resistant strains of *S. aureus* (Kon and Rai, 2012). Moreover, according to Zouhir et al. (2016), essential oils obtained from species of the Myrtaceae family were the first to demonstrate promising results against MRSA.

The resistance profile of the strains of *S. aureus* was determined by the disc diffusion test (Table 2). The antimicrobial activity of EgEO was evaluated against strains with different resistance profiles through the broth dilution test. The MIC and MBC values are shown in Table 3. The results demonstrate that EgEO exhibited inhibitory activity for all *S. aureus* strains tested, with MIC values between 0.01 and 1.84 mg/mL. Aligiannis et al. (2001) proposed a classification for plant materials based on MIC values: strong inhibition: < 0.5 mg/mL; moderate inhibition: 0.6 - 1.5 mg/mL; low inhibition > 1.6 mg/mL. Thus, EgEO demonstrated strong inhibition for six of the tested strains, mainly for strain UFPEDA 683 (MIC = 0.01 mg/mL).

MBC values were obtained for nine of the tested strains and ranged from 0.05 to > 1.84 mg/mL. The antimicrobial action of essential oils may be bacteriostatic or bactericidal. According to Gatsing et al. (2006), antimicrobial agents are considered bacteriostatic when the MBC/MIC ratio > 4 and bactericidal when the MBC/MIC ratio ≤ 4. Thus, EgEO demonstrated bactericidal action against seven strains of *S. aureus*, being more evident for the multiresistant strains (MBC/MIC = 2).

In a recent study by Chaves et al. (2018) the *Eucalyptus camaldulensis* essential oil presented antimicrobial activity against two MRSA strains, with MIC value = 1 mg/mL for both. Buldain et al. (2018) reported that the *Melaleuca armillaris* essential oil exhibited antimicrobial potential against three MRSA strains, with MIC value = 12.5 μL/mL for all. These results, as well as those obtained in our study, demonstrate the antimicrobial potential of essential oils obtained from plants of the Myrtaceae family against MRSA.

Some of the *S. aureus* strains used in the broth diluition test were selected for the time-kill assay. ATCC 29213 was selected because is a standard and non-virulent strain, and UFPEDA 683 strain was selected by the high resistance profile and considering the relevant values of MIC and MBC of EgEO obtained. The concentrations of EgEO used in this assay correspond to the MIC/2, MIC and 2xMIC values for each *S. aureus* strain (ATCC 29213: 0.23, 0.46, 0.92 mg/mL, UFPEDA 683: 0.005, 0.01, 0.02 mg/mL).

The kinetic growth of the selected strains exposed to EgEO can be observed in Figure 1A, B. The time-kill assay is widely used to determine the bactericidal effect and to obtain informations on the interaction between the antimicrobial agent and the microorganism. In addition, a possible time-dependent or concentration-dependent antimicrobial activity is revealed by the time-kill assay (Chouhan et al., 2017). The results obtained in this assay

confirmed the antimicrobial activity of EgEO against *S. aureus* and showed that the treatment time and the concentration of the essential oil had influence on bacterial growth. Bacterial growth at MIC/2 concentration was similar to control in both tested strains. MIC concentration showed reduce on bacterial growth during the first 6 h of treatment, and in the following hours it was possible to observe the inhibitory action of EgEO on bacterial growth of the ATCC 29213 strain. The treatment corresponding to the concentration of 2xMIC showed the highest antimicrobial activity in the ATCC 29213 strain. In addition, at this concentration it was possible to observe the bactericidal action of EgEO between 5 and 6 h of treatment.

Tangjitjaroenkun et al. (2012), described the bactericidal activity of the *Zanthoxylum limonella* essential oil against *S. aureus*, and noted that the treatment time for the standard strain was significantly lower when compared to the MRSA strain. Xu et al. (2016), reported that the *Syringa yunnanensis* essential oil shows antimicrobial activity against *S. aureus*, and that the antimicrobial action depends on concentration and time. In the mentioned studies, treatment with the concentration of 2xMIC was the most effective. These results corroborate our findings, evidencing the importance of treatment time and essential oil concentration.

Staphyloxanthin (STX) is a golden carotenoid pigment present in the cell membrane of *S. aureus*. Considered an important virulence factor with antioxidant properties, the pigment provides integrity to the cell membrane and promotes resistance to reactive oxygen species (ROS) generated by host neutrophils. STX has been associated with increased survival of *S. aureus* under stressful environmental conditions and during infections. Thus, inhibition in STX biosynthesis has been investigated as a possible strategy for the treatment of infections caused by *S. aureus* (Tiwari et al., 2018; Rubini et al., 2018a).

In our study, staphyloxanthine biosynthesis was reduced in *S. aureus* strains UFPEDA 02, UFPEDA 683 and UFPEDA 802 at sub-MIC and MIC concentrations of EgEO (Figure 2). The concentrations of EgEO used in this assay correspond to the MIC/8, MIC/4, MIC/2 and MIC values for each *S. aureus* strain tested (ATCC 29213: 0.05, 0.11, 0.23, 0.46 mg/mL, UFPEDA 02: 0.23, 0.46, 0.92, 1.84 mg/mL, UFPEDA 802: 0.02, 0.05, 0.11, 0.23 mg/mL and UFPEDA 683: 0.001, 0.002, 0.005, 0.01 mg/mL). According to previous studies, essential oils and their compounds can reduce or eliminate STX biosynthesis, and thus decrease the virulence of *S. aureus*. Rubini et al. (2018b), reported that the essential oil of *Pogostemon heyneanus* reduced the STX biosynthesis in multiresistant strains of *S. aureus*. Kwiatkowski et al. (2015) observed that *S. aureus* colonies treated with essential oils obtained from the species

Rosmarinus officinalis L., *Carum carvi* L., and *Foeniculum vulgare* Mill., lost natural gold pigmentation characterized by the presence of STX. Kuroda et al. (2007) reported that the farnesol sesquiterpene completely suppressed the STX biosynthesis in standard and multiresistant strains of *S. aureus*. The reduction in the STX biosynthesis observed in our study demonstrates the potential anti-virulence of EgEO against *S. aureus*.

The mealworm, *Tenebrio molitor*, has been widely used as a model host in experiments involving infection by pathogenic microorganisms, especially by the immune response exhibited by these organisms (Dobson et al., 2014; Seo et al., 2016). The *in vivo* assay was performed using *T. molitor* as the model of infection.

The larvae were infected with the bacterial inoculum (ATCC 29213 and UFPEDA 683) and treated with different concentrations of EgEO (concentrations previously determined as sublethal: 1.153, 2.302, 4.612 mg/mL).

In our study, larvae were susceptible to *S. aureus* infection. The results showed that EgEO at a concentration of 4.612 mg/mL was effective against *S. aureus* infection in *T. molitor* only in the standard strain, but was not effective at the other concentrations tested (Figure 3). In addition, EgEO was not effective against infection by the clinical strain at the concentrations tested. This is the first time that the antimicrobial potential of EgEO was evaluated *in vivo*.

Conclusions

The results obtained in this study demonstrated the antimicrobial potential of EgEO against standard and multiresistant strains of *S. aureus*. In addition, the anti-virulence action of the oil was found due to its ability to reduce the STX biosynthesis by the microorganism. EgEO has shown a promising antimicrobial profile.

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Table Captions

Table 1. Chemical compounds identified in the *Eugenia gracillima* essential oil (EgEO) by GC-MS.

Table 2. Resistance profile of standard strains and clinical isolates of *Staphylococcus aureus* by disc diffusion test.

Table 3. Minimum Inhibitory Concentration (MIC-mg/mL) and Minimum Bactericidal Concentration (MBC-mg/mL) of *Eugenia gracillima* essential oil (EgEO) against *Staphylococcus aureus*.

Table 1.

	Compounds	IR[a]	IR[b]	%
1	*δ*-elemene	1335	1330	5.89
2	*α*-cubebene	1348	1346	0.22
3	*α*-copaene	1374	1374	0.84
4	*β*-cubebene	1387	1385	0.73
5	*β*-bourbonene	1387	1387	0.99
6	*β*-elemene	1389	1390	1.89
7	*β*-caryophyllene	1417	1413	3.64
8	*β*-ylangene	1419	1418	0.87
9	*β*-duprezianene	1421	1420	1.18
10	4,8-*β*-epoxy-caryophyllene	1423	1422	0.33
11	(*E*)-*α*-Ionene	1428	1416	1.18
12	*β*-copaene	1430	1429	1.07
13	*β*-gurjunene	1431	1430	0.28
14	Aromadendrene	1439	1436	1.46
15	α-neo-clovene	1452	1453	2.15
16	Allo-aromadendrene	1458	1455	16.37
17	9-epi-(E)-caryophyllene	1464	1464	1.39
18	*γ*-gurjunene	1475	1473	0.96
19	*γ*-himachalene	1481	1482	6.11
20	*α*-amorphene	1483	1483	0.90
21	Germacrene D	1484	1485	0.23
22	*δ*-Selinene	1492	1490	3.72
23	Valencene	1496	1495	14.59
24	Viridiflorene	1496	1496	5.60
25	α-Muurolene	1500	1499	0.62
26	Epozonarene	1501	1501	0.27
27	*Trans*-*β*-guaiene	1502	1502	2.45
28	*α*-chamigrene	1503	1503	7.05
29	*β*-bisabolene	1505	1506	0.77
30	Germacrene A	1508	1509	0.56
31	α-bulnesene	1509	1510	0.76
32	*δ*-Amorphene	1511	1511	1.40
33	*γ*-Cadinene	1513	1513	4.05
34	(*Z*)-*γ*-bisabolene	1514	1514	0.95
35	Cubebol	1514	1515	2.23
36	Nootkatene	1517	1518	0.30
37	7-epi-α-selinene	1520	1521	0.61
38	Trans-calamenene	1522	1523	0.52
39	*δ*-Cadinene	1522	1525	1.39
	Sesquiterpenes			97.02
	Total			97.02

[a]Linear retention indices from the literature. [b]Retention indices calculated from retention times in relation to those of a series of n-alkanes on a 30 m DB-5 capillary column.

Table 2.

Staphylococcus aureus Strains	Source	Resistance Profile
ATCC 29213	Standard Strain	-
UFPEDA 02	Standard Strain	-
UFPEDA 659	Catheter tip	OXA/NAL
UFPEDA 670	Catheter tip	AMP/ OXA/ NAL/CIP/CLI/TRI
UFPEDA 671	Bone fragmente	AMP/OXA/NAL/CIP/AMI/GEN/CLI/CLO/TET/TRI
UFPEDA 679	Surgical wound secretion	AMP/OXA/CFL/CFZ/NAL/VAN/AMI/CLI/
UFPEDA 683	Purulent exudate	AMP/OXA/CFL/CFO/CFZ/CPM/CRX/CTX/ NAL/CIP/VAN/AMI/GEN/CLI/CLO/TRI
UFPEDA 691	Catheter tip	NAL/CIP/CLO
UFPEDA 705	Surgical wound	AMP/ OXA/CFL/CFO/CPM/CRX/NAL/NIT/GEN
UFPEDA 726	Nasal secretion	AMP/OXA/CIP/GEN/CLO/TRI
UFPEDA 731	Surgical wound secretion	AMP/OXA/CFL/CFO/CRX/NAL/CIP/GEN/ CLI/CLO/TRI
UFPEDA 802	Nasal secretion	AMP/OXA/CFL/CFO/CFZ/CPM/CRX/CTX/ NAL/CIP/AMI/GEN/CLI/CLO/TET/TRI

AMP = ampicillin; OXA = oxacillin; CFL = cephalotin; CFO = cephoxitin; CFZ = cephazoline; CPM = cefepime; CRX = cefuroxime; CTX = cefotaxime; IPM = imipenem; MER = meropenem; NAL = nalidixic acid; CIP = ciprofloxacin; NIT = nitrofurantoin; VAN = vancomycin; AMI = amikacin; GEN = gentamicin; CLI = clindamycin; CLO = chloramphenicol; TET = tetracycline; TRI = trimetropim.

Table 3.

Staphylococcus aureus Strains	MIC	MBC	MBC/MIC
ATCC 29213	0.46	1.84	4
UFPEDA 02	1.84	>1.84	ND
UFPEDA 659	0.46	0.92	2
UFPEDA 670	0.23	1.84	8
UFPEDA 671	0.23	0.46	2
UFPEDA 679	0.92	1.84	2
UFPEDA 683	0.01	0.05	5
UFPEDA 691	1.84	>1.84	ND
UFPEDA 705	0.92	1.84	2
UFPEDA 726	0.92	1.84	2
UFPEDA 731	1.84	>1.84	ND
UFPEDA 802	0.23	0.46	2

ND: Not Determined. MIC and MBC = mg/mL.

Figures Captions

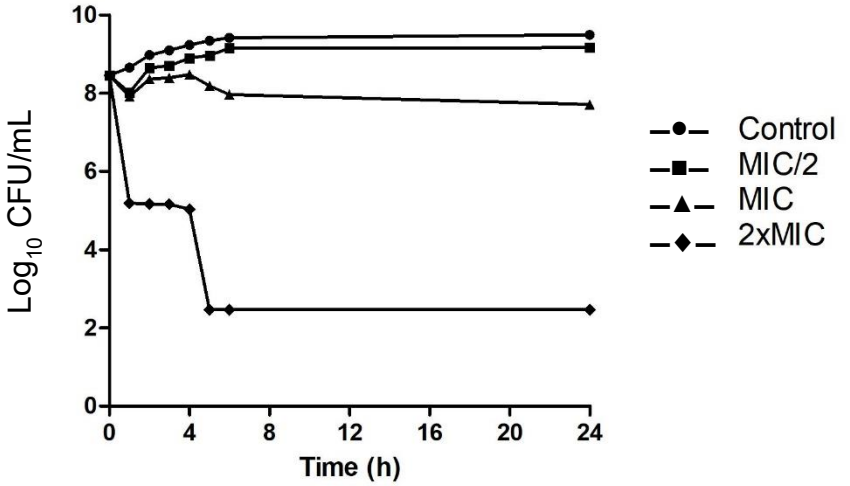
Figure 1 - Time-kill curves of *Staphylococcus aureus* ATCC 29213 (A) and UFPEDA 683 (B) after exposure to the *Eugenia gracillima* essential oil (EgEO). Control represents the untreated *Staphylococcus* cells.

Figure 2 - Profile of the staphyloxantin biosynthesis by *Staphylococcus aureus* UFPEDA 02 (A), ATCC 29213 (B), UFPEDA 683 (C) and UFPEDA 802 (D) in the presence of the respectives MIC, MIC/2, MIC/4 and MIC/8 of *Eugenia gracillima* essential oil (EgEO).

Figure 3 - Percent survival of the *Tenebrio molitor* after infection by *Staphylococcus aureus* ATCC 29213 (A) and UFPEDA 683 (B) followed by treatment with *Eugenia gracillima* essential oil (EgEO).

Figure 1

(A)


Timekill ATCC 29213
Log10 CFU/mL
Time (h)
Control
MIC/2
MIC
2xMIC

(B)

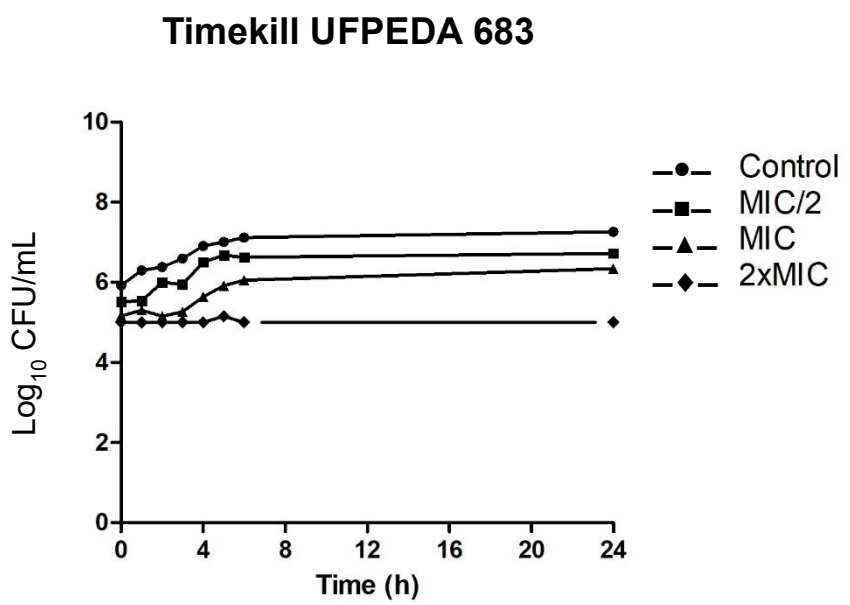
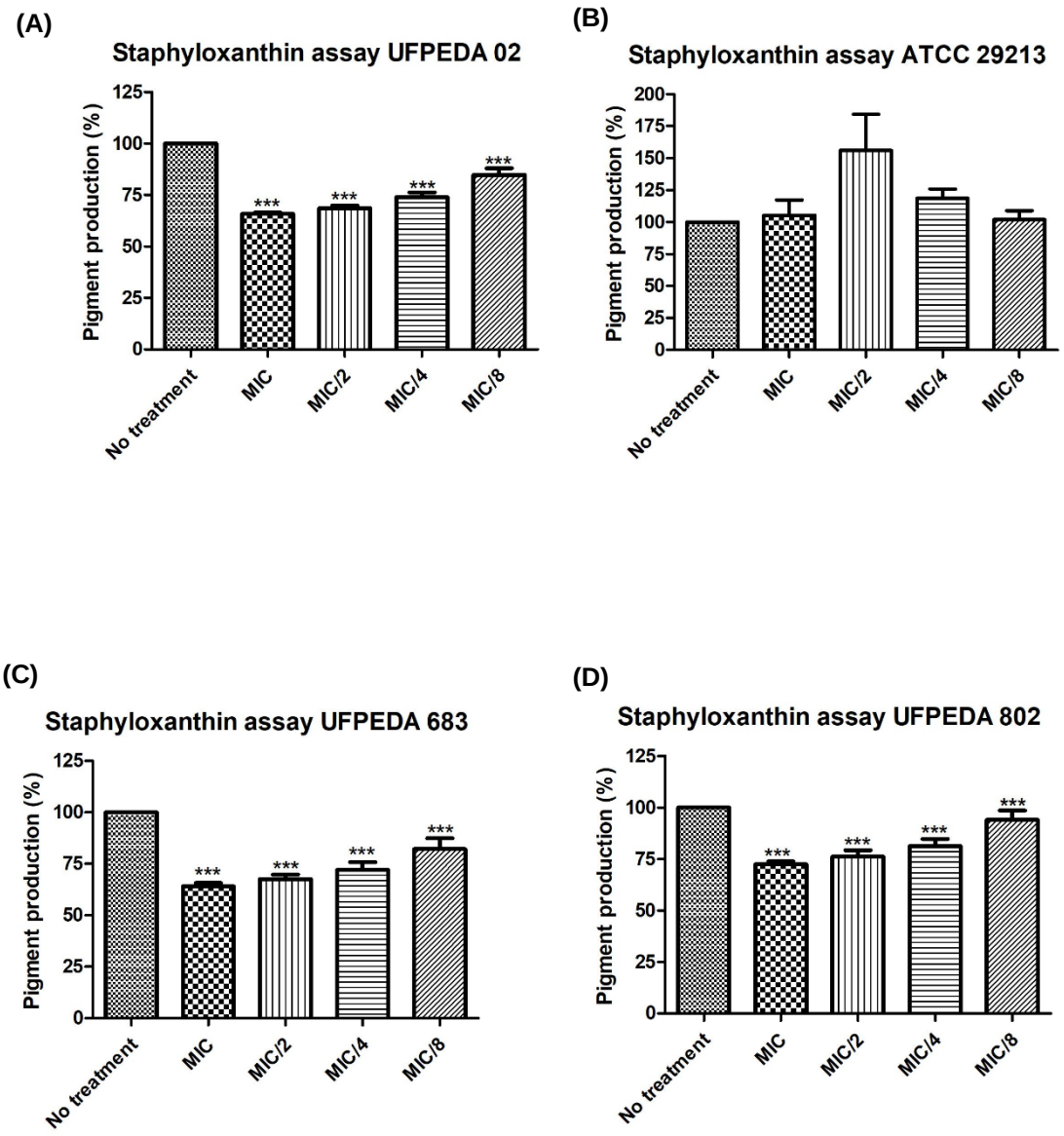
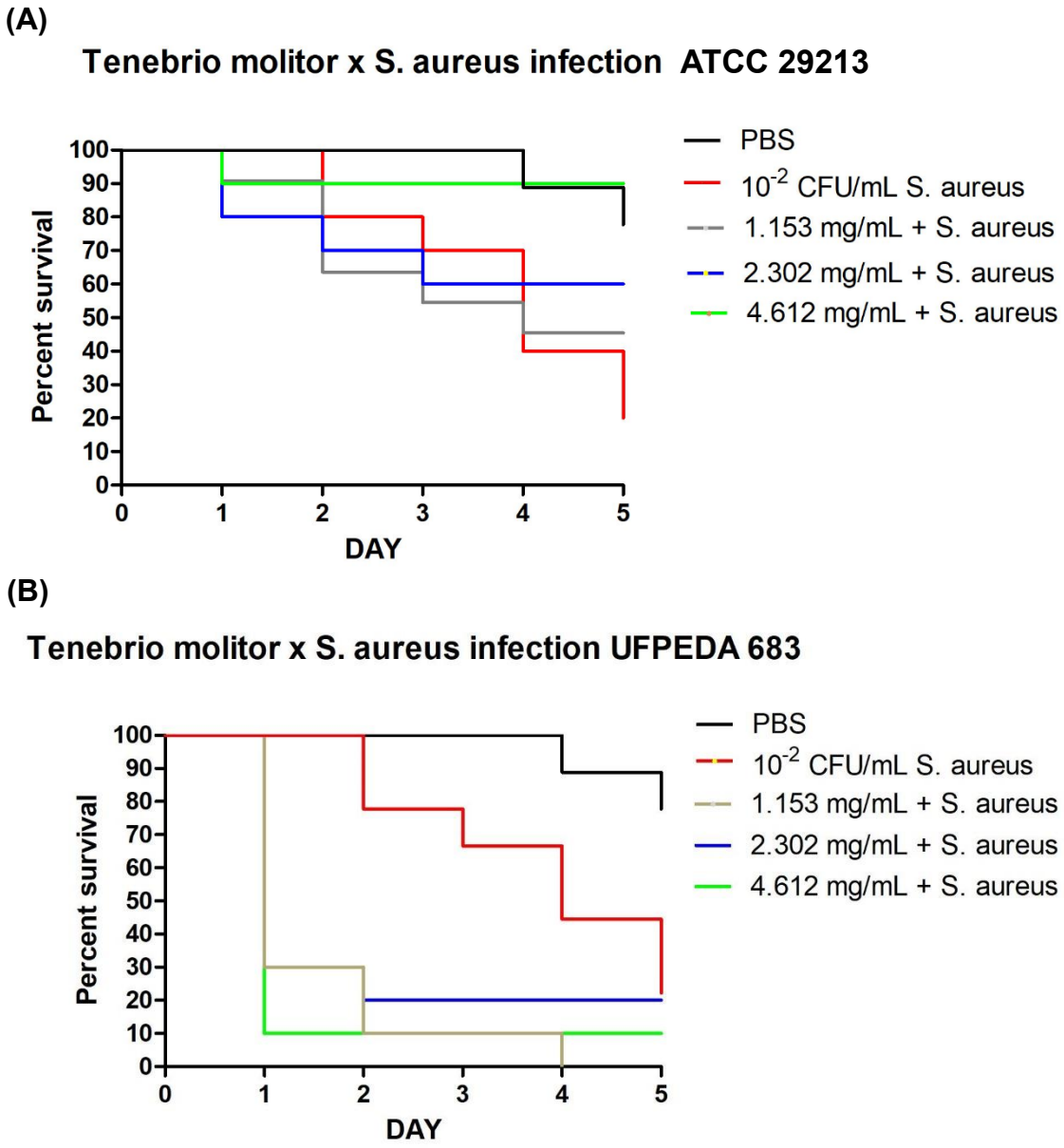

Timekill UFPEDA 683
Log10 CFU/mL
Time (h)
Control
MIC/2
MIC
2xMIC

Figure 2



*** Indicates a statistically significant difference ($p \leq 0.001$) for a comparison between *Staphylococcus aureus* incubated with PBS (No treatment) and *Staphylococcus aureus* incubated in the presence of *Eugenia gracillima* (EgEO), as determined by ANOVA.

Figure 3


(A)
Tenebrio molitor x S. aureus infection ATCC 29213
Percent survival
DAY
PBS
10-2 CFU/mL S. aureus
1.153 mg/mL + S. aureus
2.302 mg/mL + S. aureus
4.612 mg/mL + S. aureus
(B)
Tenebrio molitor x S. aureus infection UFPEDA 683
Percent survival
DAY
PBS
10-2 CFU/mL S. aureus
1.153 mg/mL + S. aureus
2.302 mg/mL + S. aureus
4.612 mg/mL + S. aureus

3.3 ARTIGO 3

ANTIFUNGAL ACTIVITY OF *Eugenia gracillima* KIAERSK. ESSENTIAL OIL AGAINST *Candida glabrata*: *in vitro* AND *in vivo* EVALUATION

Artigo a ser submetido ao periódico **Microbiological Research**

(**FI:** 2.777; **QUALIS CB I:** B1)

Antifungal activity of *Eugenia gracillima* Kiaersk. essential oil against *Candida glabrata*: *in vitro* and *in vivo* evaluation

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Abstract

Candida glabrata is an emerging opportunistic pathogen. Its ability to rapidly develop resistance to antifungal agents has been associated with an increase in its prevalence. The aim of this study was to analyze the chemical composition and *in vitro* and *in vivo* effects of *Eugenia gracillima* essential oil (EgEO) on standard and clinical strains of *C. glabrata*. The EO was obtained from the leaves of the species by the hydrodistillation method and its chemical composition analyzed by GC/MS. The *in vitro* antifungal susceptibility test was performed by the microdilution method. The kinetics of fungal death was determined by the time kill assay. *In vivo* antifungal activity was evaluated using *T. molitor* as the model of infection. All compounds identified in EgEO belong to the sesquiterpene class. EgEO efficiently inhibited the growth of all strains. The minimum inhibitory concentration (MIC) range was 0.11 to 3.69 mg/mL and the minimum fungicide concentration (MFC) range was 0.92 to 3.69 mg/mL. Time-kill curves showed that the oil at concentrations corresponding to MIC and 2xMIC values was considered fungistatic and fungicidal, respectively. At a concentration previously determined as sublethal (2.302 mg/mL), the essential oil was effective against *C. glabrata* infection in *T. molitor* only in the standard strain. EgEO has relevant antifungal potential against *C. glabrata* and may be a promising therapeutic agent.

Keywords: Myrtaceae. Sesquiterpenes. Time-kill assay. *Tenebrio molitor*.

Introduction

Candida spp. are considered important opportunistic pathogens that can cause local or systemic infections. They are among the major pathogens related to nosocomial infections, especially in patients admitted to an intensive care unit (ICU) (Vahedi et al., 2015). Although *C. albicans* is the most common species associated with candidiasis, non-*albicans Candida* (NAC) species, including *C. glabrata*, *C. tropicalis* and *C. parapsilosis* are often responsible for these infections (Vallabhaneni et al., 2016).

C. glabrata emerged as a pathogen that quickly developed resistance to multiple drugs, leaving no treatment options in some cases. Resistance to different classes of drugs that have distinct mechanisms of action, such as triazoles and echinocandins, has been developed over a shorter period of time compared to any other *Candida* species (Cavalheiro et al., 2019; Healey et al., 2016). In addition to the multidrug resistance presented by the pathogen, the high toxicity, drug interactions, and insufficient bioavailability of active ingredients of the available antifungal contribute to therapeutic failure (Vale-Silva; Sanglard, 2015).

The increased prevalence of candidiasis caused by *C. glabrata* has been attributed to the increasing number of multidrug resistant strains and the shortage of new classes of antifungals. Therefore, in search of new therapeutic options, many compounds found in plants have been investigated (Kalidindi et al, 2015). Essential oils (EOs) from aromatic plants have bioactive compounds with antifungal properties, including anti-*Candida*, and may be new antifungal agents used in the treatment of candidiasis (Sharifi-Rad et al., 2017).

The genus *Eugenia* (Myrtaceae) comprises about 1100 species, of which more than 380 are found in Brazil, where many species have commercial and nutritional importance attributed to the production of edible fruits. In addition, like other Myrtaceae, most species of the genus are rich in EOs that may exhibit aromatic and biological properties. The EOs of *Eugenia* have a remarkable chemical diversity, with a high number of distinct compounds identified in the analyzed species, being predominant cyclic sesquiterpenes and monoterpenes (Bida et al., 2018).

E. gracillima Kiaersk., popularly known as “murta”, is a fructiferous shrub, native and endemic to Brazil. Its fruits have important nutritional value attributed to the high content of anthocyanins, and can be consumed fresh or processed. The species is economically important

in northeastern Brazil, particularly in the Chapada do Araripe (Pernambuco, Brazil), where products such as pulps, jellies and liqueurs are marketed (Feitosa et al, 2018).

The aim of this study was to evaluate the chemical composition and antifungal potential *in vitro* and *in vivo* of the *E. gracillima* essential oil (EgEO) against *Candida glabrata*.

Materials and Methods

Plant Material

Eugenia gracillima Kiaersk., was collected in the Chapada do Araripe, Pernambuco, Brazil (Latitude- 7° 49' 41" S; Longitude- 39° 51' 18" W), in July 2015. All the material used was processed following the usual techniques in taxonomy, being deposited in the IPA Herbarium (voucher access number: IPA 91.440), from the Agronomic Institute of Pernambuco.

Essential Oil Extraction

Essential oil extraction was performed at the Laboratory of Natural Products, Federal University of Pernambuco, Brazil. Fresh leaves of *E. gracillima* (370 g) were submitted to the hydrodistillation method, using a modified Clevenger apparatus, for a 4 h period (Guimarães et al., 2008). The essential oil of *E. gracillima* (EgEO) was then separated from the hydrolate by centrifugation at 3000 rpm for 5 min, using a benchtop centrifuge. EgEO was collected, packed in amber glass bottle and stored under refrigeration (4 °C). The EgEO yield was 1.32%.

Chemical Analysis of the Essential Oil

Gas chromatography

Quantitative GC analyses were carried out using a Shimadzu GC-QP2010 Ultra apparatus equipped with a flame ionization detector (FID) and a non-polar column Rxi®-5ms (Crossbond® 5% diphenyl / 95% dimethylpolysiloxane) (10m x 0,10 mm ID x 10µm df). The oven temperature was programmed from 40 to 250°C at a rate 25°C/min for integration purposes. Injector and detector temperatures were 260°C. Hydrogen was used as the carrier gas at a flow rate of 1 L/min and 30 p.s.i. inlet pressure in split mode (1:30). The injection volume was 0.5 µL of diluted solution (1/100) of oil in n-hexane. The amount of each compound was calculated from GC peak areas in the order of DB-5 column elution and expressed as a

relative percentage of the total area of the chromatograms. Analyses were carried out in triplicate.

Gas chromatography-mass spectrometry

The qualitative GC/MS analysis were carried out using a analyses were performed using a Shimadzu GCMS-QP2010 Ultra system with a mass selective detector, mass spectrometer in EI 70 eV with a scan interval of 0.5 s and fragments from 40 to 550 Da. fitted with the same column and temperature program as that for the GC experiments, with the following parameters: carrier gas = helium; flow rate = 1 mL/min; split mode (1:30); injected volume = 1 µL of diluted solution (1/100) of oil in n-hexane.

Identification of componentes

Identification of the components was based on GC retention indices with reference to a homologous series of C8-C40 n-alkanes calculated using the Van Den Dool and Kratz equation (Van Den Dool and Kratz, 1963) and by computer matching against the mass spectral library of the GC/MS data system (NIST 98 and WILEY) and co-injection with authentic standards as well as other published mass spectra (Adams, 2007). Area percentages were obtained electronically from the GC-FID response without the use of an internal standard or correction factors.

Antifungal Activity

Fungal strains

Strains of *Candida glabrata* ATCC 2001 and clinical isolates: URM 4246, 4247, 4264, 5664, 5933, 6393 were obtained from the Fungal Culture Collection Micoteca URM (Department of Mycology, Federal University of Pernambuco, Recife, Brazil). All strains were maintained on Sabouraud dextrose agar at 4°C until used in antifungal assays.

Inoculum preparation

The inoculum suspension was prepared by picking five colonies of ~ 1 mm in diameter from cultures of *Candida glabrata* strains grown on Sabouraud dextrose agar for 24 h at 35°C. The colonies were suspended in 5 mL of sterile saline solution (0.85%) and the suspension was vortexed for 15 s and the cell density was adjusted with a spectrophotometer by adding sufficient sterile saline to increase the transmittance to that produced by a 0.5 McFarland standard at 530 nm wavelength (1.0-5.0 x 10^6 cells per mL). The final inoculum suspension

was made by a 1:100 dilution followed by a 1:20 dilution of the stock suspension using RPMI 1640 (Roswell Park Memorial Institute - 1640) culture medium with MOPS (3-(N-morpholino) propanesulfonic acid), which results in 2.5-5.0 x 10^3 cells per mL.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

The evaluation of the antifungal activity was performed by the broth microdilution method according to the protocol described by Clinical and Laboratory Standards Institute (CLSI) in the M27-A3 document (CLSI, 2008). For the broth microdilution assay, RPMI 1640 medium with MOPS (100 µL) was plated in 96-well microplates. The essential oil suspension (100 µL) was added in the first column and a twofold serial dilution were made until the eighth column. The EgEO concentrations ranged from 0.02 mg/mL to 3.69 mg/mL. Finally, 100 µL of inoculum suspension was added. Amphotericin B (AmB) and fluconazole (FLU) (Sigma-Aldrich®) were used as the positive controls. Additional controls also included the sterility (drug-free medium only) and yeast growth (drug-free medium and inoculum suspension). The microplates were incubated for 48 h at 35°C and the absorbance was measured in a microplate reader at a wavelength of 492 nm.

The minimum inhibitory concentration (MIC_{50}) was defined as the lowest concentration of EgEO capable of causing ≥ 50% reduction in optical density (OD_{492}) relative to OD_{492} in negative control (yeast growth control).

The content from wells (50 µL) was subcultured on Sabouraud dextrose agar and incubated for for 48 h at 35ºC, to determine the minimum fungicidal concentration (MFC). MFC was defined as the lowest concentration capable of causing death of all yeast cells, characterized by no visible growth on the solid medium. The assays were performed in quadruplicate.

Time-kill assay

The time-kill assay was performed according to Santos-Filho et al. (2015), with modifications. To determine the kinetics of fungal death, two strains were selected: a standard strain ATCC 2001 and a clinical strain URM 4247, in accordance with the MIC and MFC results. The inoculum suspension (100 µL; 2.5 x 10^3 CFU/mL) was resuspended in 900 µL of RPMI 1640 medium buffered with MOPS containing different concentrations of the EgEO (MIC/2, MIC, and 2xMIC) and incubated at 35 °C on an shaker at 200 rpm. At predetermined

time points (0, 1, 2, 3, 4, 8, 12, 24 and 48 h) 100 µL aliquots were removed from each solution. The aliquots were diluted in sterile saline solution and 30 µL were plated on SDA. Colonies were counted after incubation at 35 °C for 48 h. The yeast growth control was performed (negative control). The experiment was performed in triplicate.

In vivo assays

Tenebrio molitor

T. molitor larvae were purchased from a commercial supplier (Insetos Brasil – Recife, Pernambuco, Brazil) and kept in plastic trays (60 cm long×40cm wide×12cm) at 26 ºC ± 1 in a 12:12 h light/dark cycle. These individuals were fed *ad libitum* with wheat bran (12% protein, 2% lipids, 75% carbohydrates and 11% mineral/sugar), pieces of sugarcane and chayote. Healthy larvae (last instar larval) were chosen for the bioassays.

T. molitor x *C. glabrata* infection

C. glabrata was cultured in RPMI 1640 medium with MOPS overnight at 37°C. The cells were centrifuged at 3500 rpm for 10 min. The bacterial pellet was washed twice in phosphate-buffered saline (PBS) and diluted with PBS to reach a concentration of $2.5x10^2$ cells/mL. *T. molitor* larvae were microinjected with 1 µL suspensions of *C. glabrata* (10^2 cells/mL) using a 1 mL syringe. After 2 h of infection, the larvae were treated with EgEO (concentrations previously determined as sublethal: 1.153, 2.302, 4.612 mg/mL). The control larvae were injected with 1 µL of PBS at the same time. Following this, larvae were maintained under standard rearing conditions in isolation. A total of 30 larvae per treatment (n = 10) were used and the results represent an average of three biological replications.

Statistical Analysis

Data were expressed as mean ± standard deviation. Statistical analysis and graphical representation of the data were performed using *GraphPad Prism* 7.0 (GraphPad Software). Data were analyzed using ANOVA. P values < 0.05 were considered statistically significant.

Results and Discussion

Candida spp. cause mucosal, disseminated and invasive candidiasis, especially among patients who are immunecompromised or hospitalized with serious underlying diseases. The

overall mortality for invasive infections caused by *Candida* spp. requires the emergence of new therapeutic strategies.

The chemical composition of the essential oil from *E. gracillima* leaves is shown in Table 1. The main constituents of EgEO were allo-aromadendrene (16.37%), valencene (14.59%), α-chamigrene (7.05%), γ-himachalene (6.11%), δ-elemene (5.89%) and viridiflorene (5.60%). A total of 39 components were identified in the oil, accounting for 97.02% of the total composition. All compounds identified belong to the sesquiterpene class.

The antifungal activity of the EgEO is shown in Table 2. The results show that the EgEO had effective antifungal activity with a MIC range of 0.11 – 1.84 mg/mL, including for isolates resistant to amphotericin-B and fluconazole. Furthermore, the EO showed fungicidal activity against all fungi except three clinical isolates of *C. glabrata* that were resistant to the EO with MIC > 3.69 mg/mL.

Essential oils containing high content of sesquiterpenes have demonstrad good results against *C. glabrata*. The essential oil of *Kundmannia syriaca* fruits composed mainly of sesquiterpenes (60.3% of 74.8% of identified compounds) possess antifungal activity against standard strains of five different human pathogenic *Candida* species, including *C. glabrata* (MIC value of 62.5 μg/mL) (Kaya et al., 2016). The essential oil from leaves of *Casearia sylvestris* rich in non-oxygenated sesquiterpenes (72.1%) and its fraction rich in oxygenated sesquiterpenes were active against standard strain of *C. glabrata*, presented MIC value of 125 μg/mL for both (Pereira et al., 2017).

Although the studies cited above show excellent results of antifungal activity against *C. glabrata*, the essential oils were evaluated only in standard strains. In our study, EgEO was effective against standard and clinical strains.

Previous studies have reported that sesquiterpenes induce changes in cell permeability, disrupting lipid packing and causing damage to membrane properties and functions. In addition, sesquiterpenes are also related to disorders of mitochondrial membrane in *Candida* species (Nazzaro et al., 2017; Elhidar et al., 2019).

Sesquiterpene compounds or compositions comprising one or more sesquiterpene compounds as active ingredients have been proposed for the treatment of infections caused by a large number of pathogenic fungi, including *C. glabrata*. These compounds may be administered by

various means including oral, topical, inhalation, intravenous or parenteral administration (Vediyappan, Hua, 2015).

Strains of *C. glabrata* (ATCC 2001 and URM 4247) were submitted to the experimental method of microbial death kinetics. Yeast growth was analyzed over time while undergoing different concentrations of EgEO, corresponding to MIC/2, MIC and 2xMIC values (ATCC 2001: 0.23, 0.46, 0.92 mg/mL and URM 4247: 0.05, 0.11, 0.22 mg/mL).

The graphs show the $\log_{10}$ of CFU/mL versus time of exposure in the presence of EgEO and the growth control (Figure 1). The results showed that EgEO inhibited the fungal growth of the different strains in a similar manner. Analysis of the graphs reveals that the EgEO concentration MIC has fungistatic activity, since there was a reduction of the fungal growth at up to 2 h of exposure when compared to the control; this behavior was also seen for the MIC/2 but in much smaller reach. At the concentration of 2xMIC, fungicidal activity was observed (100% inhibition in *C. glabrata* viability of the initial inoculum). Therefore, it was possible to identify in the death kinetics a transition between fungistatic and fungicidal activity, revealing concentration-dependent fungicidal activity.

Time-kill assay analyzes the interaction of a test product with microorganisms to characterize a dynamic relationship between concentration and activity over time. The test reveals a time-dependent or a concentration-dependent an antimicrobial effect (Balouiri et al., 2016).

The *in vivo* assay was performed using *Tenebrio molitor* as the model of infection. The larvae were infected with the fungal inoculum (ATCC 2001 and URM 4247) and treated with different concentrations of EgEO (concentrations previously determined as sublethal: 1.153, 2.302, 4.612 mg/mL). The results showed that EgEO at a concentration of 2.302 mg/mL had potential to combat infection by the standard strain, but was not effective at the other concentrations tested. In addition, EgEO had no potential to combat infection by the clinical strain at the concentrations tested (Figure 2).

T. molitor has some important advantages when compared to other alternative models of invertebrate hosts used in the study of human fungal pathogens. The mealworm can be incubated at 37 ºC which, for many pathogens, is a necessary environmental condition for the expression of virulence factors. Another advantage is that the inoculum is administered by injection, allowing the direct introduction into the hemolymph of the larva. In addition, dead

larvae are easily identified because they become brown by melanization (Canteri de Souza et al., 2018).

De Souza et al. (2015) evaluated the utility of *T. molitor* as a model host to study infections caused by *Candida albicans* and *Cryptococcus neoformans*, infecting the larvae with different concentrations of the pathogens and verifying mortality. The results showed that larval survival decreased proportionally to the increase of inoculum concentration, similar behavior to that of *Galleria mellonella* and mice with these yeasts.

Morey et al. (2016) used *T. molitor* as model to verify the efficacy of a potential antifungal agent. In this study, the larvae of *T. molitor* were infected with *Candida tropicalis*, and treated with a tannin-rich fraction from a *Stryphnodendron adstringens* extract. The survival of the larvae that received the inoculum containing the fraction of the plant extract was significantly higher compared to the untreated group, indicating not only that the larvae are sensitive to *C. tropicalis* infection, but can also be used as an alternative host model to evaluate the activity of potential antifungal compounds.

Our study demonstrated that larvae were susceptible to *C. glabrata* infection. We do not know of any studies that report the evaluation of a potential antifungal agent using *T. molitor* as a model to *C. glabrata* infection.

Conclusions

According to the results of this study, it is possible to conclude that EgEO is a promising source of active molecules with antifungal properties. The biological assays reported in this investigation show that the EgEO inhibits standard and clinical strains of *C. glabrata*, including those with resistance to drugs employed in medical practice.

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Table Captions

Table 1. Chemical compounds identified in the *Eugenia gracillima* essential oil (EgEO) by GC-MS.

Table 2. Minimum Inhibitory Concentration (MIC-mg/mL) and Minimum Fungicidal Concentration (MFC-mg/mL) of *Eugenia gracillima* essential oil (EgEO) against *Candida glabrata*.

Table 1.

	Compounds	IR[a]	IR[b]	%
1	*δ*-elemene	1335	1330	5.89
2	*α*-cubebene	1348	1346	0.22
3	*α*-copaene	1374	1374	0.84
4	*β*-cubebene	1387	1385	0.73
5	*β*-bourbonene	1387	1387	0.99
6	*β*-elemene	1389	1390	1.89
7	*β*-caryophyllene	1417	1413	3.64
8	*β*-ylangene	1419	1418	0.87
9	*β*-duprezianene	1421	1420	1.18
10	4,8-*β*-epoxy-caryophyllene	1423	1422	0.33
11	(*E*)-*α*-Ionene	1428	1416	1.18
12	*β*-copaene	1430	1429	1.07
13	*β*-gurjunene	1431	1430	0.28
14	Aromadendrene	1439	1436	1.46
15	α-neo-clovene	1452	1453	2.15
16	Allo-aromadendrene	1458	1455	16.37
17	9-epi-(E)-caryophyllene	1464	1464	1.39
18	*γ*-gurjunene	1475	1473	0.96
19	*γ*-himachalene	1481	1482	6.11
20	*α*-amorphene	1483	1483	0.90
21	Germacrene D	1484	1485	0.23
22	*δ*-Selinene	1492	1490	3.72
23	Valencene	1496	1495	14.59
24	Viridiflorene	1496	1496	5.60
25	α-Muurolene	1500	1499	0.62
26	Epozonarene	1501	1501	0.27
27	*Trans*-*β*-guaiene	1502	1502	2.45
28	*α*-chamigrene	1503	1503	7.05
29	*β*-bisabolene	1505	1506	0.77
30	Germacrene A	1508	1509	0.56
31	α-bulnesene	1509	1510	0.76
32	*δ*-Amorphene	1511	1511	1.40
33	*γ*-Cadinene	1513	1513	4.05
34	(*Z*)-*γ*-bisabolene	1514	1514	0.95
35	Cubebol	1514	1515	2.23
36	Nootkatene	1517	1518	0.30
37	7-epi-α-selinene	1520	1521	0.61
38	Trans-calamenene	1522	1523	0.52
39	*δ*-Cadinene	1522	1525	1.39
	Sesquiterpenes			97.02
	Total			97.02

[a]Linear retention indices from the literature. [b]Retention indices calculated from retention times in relation to those of a series of n-alkanes on a 30 m DB-5 capillary column.

Table 2.

Candida glabrata Strains	Source	MIC_{50} (mg/mL)	MFC (mg/mL)	AmB* MIC (µg/mL)	FLU* MIC (µg/mL)
ATCC 2001 (S)	Standard Strain	0.46	1.84	0.5	4
URM 4246 (S)	Blood	3.69	>3.69	0.25	1
URM 4247 (S)	Blood	0.11	0.92	0.5	2
URM 4264 (R)	Oropharyngeal secretion	0.92	3.69	4	> 64
URM 5664 (R)	Blood	1.84	3.69	2	> 64
URM 5933 (S)	Blood	3.69	>3.69	0.06	0.5
URM 6393 (S)	Blood	3.69	>3.69	0.25	2

*AmB: Amphotericin B; FLU: Fluconazole. (S): sensitive; (R): resistant.

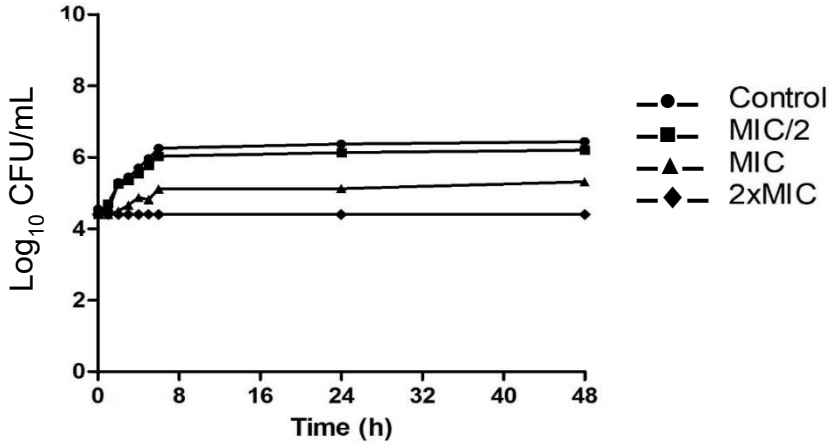
Figures Captions

Figure 1 - Time-kill curves of *Candida glabrata* ATCC 2001 (A) and URM 4247 (B) following exposure to the *Eugenia gracillima* essential oil (EgEO). Control represents the untreated *Candida* cells.

Figure 2 - Percent survival of the *Tenebrio molitor* after infection by *Candida glabrata* ATCC 2001 (A) and URM 4247 (B) followed by treatment with *Eugenia gracillima* essential oil (EgEO).

Figure 1

(A)


Timekill ATCC 2001
Log10 CFU/mL
Time (h)
Control
MIC/2
MIC
2xMIC

(B)

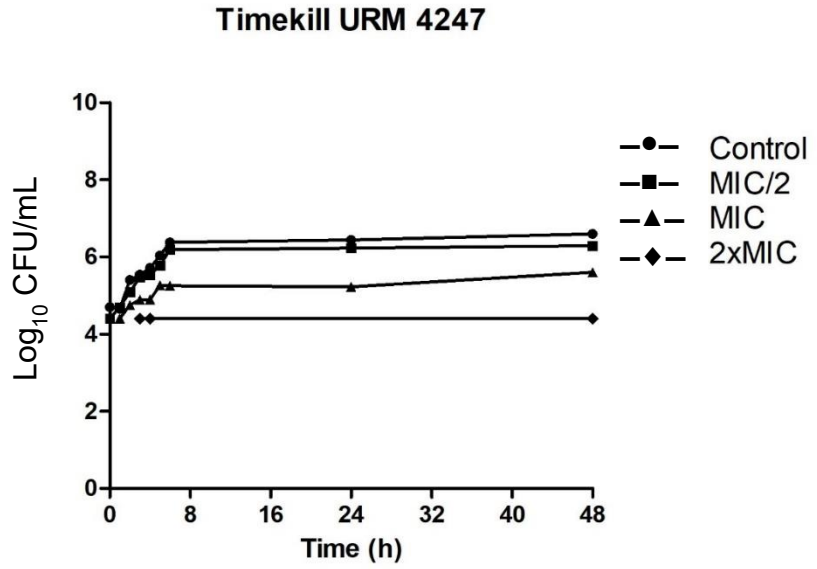
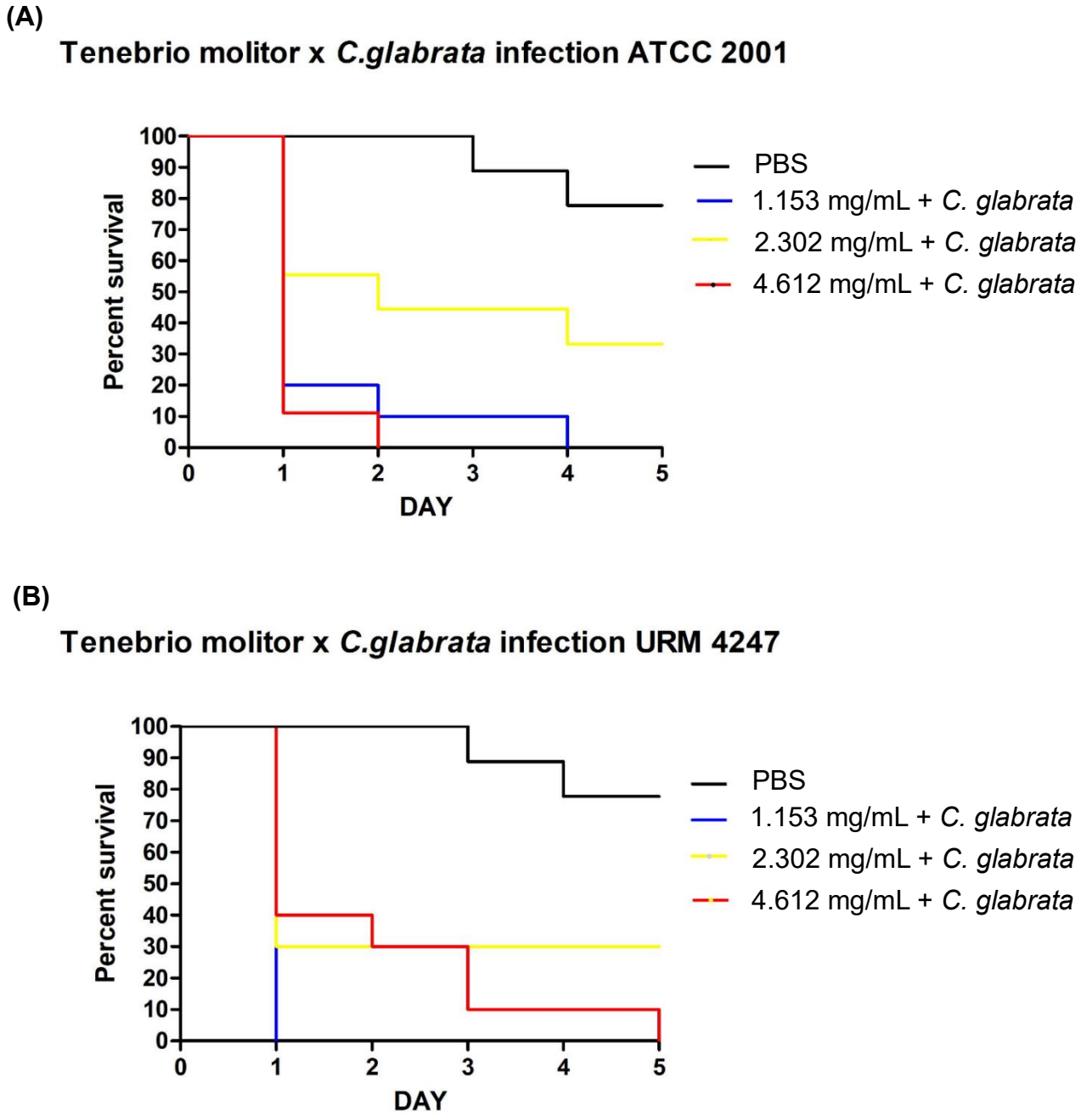

Timekill URM 4247
Log10 CFU/mL
Time (h)
Control
MIC/2
MIC
2xMIC

Figure 2


(A)
Tenebrio molitor x C.glabrata infection ATCC 2001
Percent survival
DAY
PBS
1.153 mg/mL + C. glabrata
2.302 mg/mL + C. glabrata
4.612 mg/mL + C. glabrata
(B)
Tenebrio molitor x C.glabrata infection URM 4247
Percent survival
DAY
PBS
1.153 mg/mL + C. glabrata
2.302 mg/mL + C. glabrata
4.612 mg/mL + C. glabrata

4 CONCLUSÃO

- Os dados obtidos neste trabalho atribuem ao óleo essencial das folhas de *Eugenia gracillima* relevante potencial anti-*Staphylococcus aureus* e anti-*Candida glabrata*, dois importantes patógenos oportunistas associados à resistência aos antimicrobianos atualmente disponíveis;

- A análise química demonstrou que os compostos identificados no óleo pertencem a classe dos sesquiterpenos, conhecidos por suas diversas propriedades biológicas;

- O óleo essencial demonstrou baixa toxicidade *in vitro* para eritrócitos humanos e *in vivo* para *Tenebrio molitor*;

- O óleo essencial de *E. gracillima* apresenta um perfil antimicrobiano promissor, podendo tornar-se um fármaco utilizado para o tratamento de infecções causadas por micro-organismos multirresistentes.

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ANEXO A – AUTHOR GUIDELINES

JOURNAL OF APPLIED TOXICOLOGY

Journal of Applied Toxicology

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Aims and Scope

Journal of Applied Toxicology publishes peer-reviewed original reviews and hypothesis-driven research articles on mechanistic, fundamental and applied research relating to the toxicity of drugs and chemicals at the molecular, cellular, tissue, target organ and whole body level *in vivo* (by all routes of exposure) and *in vitro / ex vivo*. All aspects of toxicology are covered (including but not limited to nanotoxicology, genomics and proteomics, teratogenesis, carcinogenesis, mutagenesis, reproductive and endocrine toxicology, toxicopathology, target organ toxicity, systems toxicity (eg immunotoxicity), neurobehavioral toxicology, mechanistic studies, biochemical and molecular toxicology, novel biomarkers, pharmacokinetics/PBPK, risk assessment and environmental health studies) and emphasis is given to papers of clear application to human health and/or provide significant contributions and impact to their field.

In addition *Journal of Applied Toxicology* also publishes analytical toxicology, ecotoxicology (vertebrate laboratory or field studies), new techniques and method development studies, (including research on animal alternatives and *in silico* modelling) and applied regulatory

toxicology on novel or existing drugs and chemicals, addressing novel, important or topical aspects of toxicology.

Reviews, mini-reviews and hypothesis/theoretical papers on any aspect of toxicology including individual chemicals and multidisciplinary subjects are welcomed (see specific instructions below).

High quality histopathology photomicrographs, specimen photographs and complex diagrams (including gene regulation) will be printed in color free to authors at the editors discretion.

Authors wishing to submit a manuscript on chemical weapons material's must contact the Editor-in-Chief first to discuss merit and scope.

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Research Articles. The **experimental section** must be precise and give all details necessary for repeating the work. Accurate and detailed descriptions must be given of the methods used. Particular attention should be given to chemicals and reagents used (supply, constitution, purity, etc) and to full descriptions of animals and their housing and environmental conditions (strain, supply, weight, caging including size and numbers per cage, ambient temperature, humidity, lighting regime, and food and water supplies). Similar detail should be provided for *ex vivo* and *in vitro* studies including derivation of tissue and cell lines, incubation and treatment procedures. See section on “Presentation of papers”.

Short Communications should not exceed two printed pages in length, including a short abstract.

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or class, allowed environmental levels etc) provided appropriate discussion and context is given. A minireview may be a more recent or limited update of a previous substantial review.

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Example of reference with 2 to 7 authors

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Ramus, F., Rosen, S., Dakin, S. C., Day, B. L., Castellote, J. M., White, S., & Frith, U. (2003). Theories of developmental dyslexia: Insights from a multiple case study of dyslexic adults. *Brain, 126*(4), 841–865. doi: 10.1093/brain/awg076

Example of reference with more than 7 authors

Rutter, M., Caspi, A., Fergusson, D., Horwood, L. J., Goodman, R., Maughan, B., … Carroll, J. (2004). Sex differences in developmental reading disability: New findings from 4 epidemiological studies. *Journal of the American Medical Association, 291*(16), 2007–2012. doi: 10.1001/jama.291.16.2007

Example of other references

van Bergen, E., de Jong, P. F., Maassen, B., Krikhaar, E., Plakas, A., & van der Leij, A. (in press). IQ of four-year-olds who go on to develop dyslexia. *Journal of Learning Disabilities*. doi: 10.1177/0022219413479673

Howell, K. W., Fox, S. L., & Morehead, K. W. (1993). *Curriculum-based evaluation: Teaching and decision making* (2nd ed.). Pacific Grove, CA: Brooks/Cole Publishing Company.

Fan, K. Y. (1986, September). *Graphic symbol of the Chinese character*. Paper presented at the meeting of the Symposium of Chinese Character Modernization, Beijing, China.

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ANEXO B – GUIDE FOR AUTHORS

MICROBIOLOGICAL RESEARCH

Microbiological Research

Guide for Authors

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